

**REMARKS**

Reconsideration of this application is respectfully requested.

Claims 35-46 and 48-50 have been canceled. Upon amendment, claim 47 is pending in this application.

Claims 35, 37, 39, 41, 43, and 45 were rejected under 35 U.S.C. § 102(e) as allegedly being anticipated by Chang (U.S. Patent No. 6,001,977).

Applicants traverse the rejection for the reasons set forth in applicants' April 23, 2004, Amendment and Response. Nonetheless, to expedite prosecution, applicants have canceled claims 35, 37, 39, 41, 43, and 45. Accordingly, this rejection is moot.

Claims 35-50 were rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Chang et al. in view of White et al. (U.S. Patent No. 4,677,054).

Applicants traverse the rejection for the reasons set forth in applicants' April 23, 2004, Amendment and Response. Nonetheless, to expedite prosecution, applicants have canceled claims 35-46 and 48-50, and will focus this response on claim 47.

Claim 47 is a method for detecting HIV-1 and is fully supported in the present application and through the priority chain now claimed to Appln. Ser. No. 06/558,109, filed **December 5, 1983**. The earliest claimed priority date of Chang is August 22, 1984. Consequently, Chang is not effective prior art with respect to claim 47.

The Examiner's statements that "priority of invention to Chang has already been awarded and Applicant can no longer contest this point" and that "Chang is, per se, prior art" are not accurate with respect to the patentability of applicant's claim 47 over Chang. Interference estoppel is created by the failure of a party in an interference to move to raise an issue that could have been properly raised during the interference. The issue

of whether Chang might have priority to the subject matter of claim 47 could **not** have been properly raised by applicants during the interference because the subject matter of claim 47 would have been unpatentable to Chang under 35 U.S.C. §102(b). In May of 1983, a paper by Barre-Sinoussi et al. was published in *Science* entitled "Isolation of a T-Lymphotropic Retrovirus from a patient at Risk for Acquired Immune Deficiency Syndrome (AIDS)" describing the detection of HIV-1 nucleic acid in cell-free supernatants of infected cells. Barre-Sinoussi et al., *Science* 220:868-871 at Fig. 1 (Exhibit 1). May of 1983 is more than one year prior to Chang's earliest priority date of August 22, 1984. Thus, a claim to the same subject matter as applicants' claim 47 would have been unpatentable to Chang under 35 U.S.C. §102(b). Consequently, applicants could not have properly raised the issue of priority with respect to the subject matter of claim 47. Accordingly, applicants respectfully request withdrawal of the rejection.

Claim 47 was rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter that was not described in the specification in such a way as to reasonably convey that the inventors had possession of the claimed invention at the time the application was filed. It is the Examiner's position that applicants have only disclosed a single species of a large genus, and that the claim does not indicate any common attributes of the sequences.

Applicants traverse the rejection. Claim 47 is a claim for a method of detecting the presence of nucleic acid of a Human Immunodeficiency Virus Type 1 (HIV-1) in a biological sample. Applicants discovered that HIV-1 was a retrovirus (i.e., an extracellular RNA genome) and how to prepare and detect the presence of HIV-1

nucleic acid. Applicants' claimed method does not require a specific sequence for detection and will work with all species of HIV-1. Many different well-known techniques could be used in the claimed method. As objective evidence of well-known techniques in the art for detecting viral RNAs at the time the application was filed, applicants provide herewith 11 exhibits.

For example, Strickland et al., 1977 (Exhibit 2), detected retroviral RNA using reverse transcriptase and oligo(dT). Strickland et al. at 343-346. Tracy et al., 1980 (Exhibit 3), detected retroviral RNA by hybridization with a [<sup>3</sup>H]poly(dT) probe. Tracy et al. at 3793-3798. Cavalieri, 1974 (Exhibit 4), detected retroviral RNA by fluorescence measurements of the intercalation of ethidium bromide. Cavalieri at 1458-1461. Herring et al., 1982 (Exhibit 5), and Berry et al., 1982 (Exhibit 6), detected viral RNAs by polyacrylamide gel electrophoresis and silver-staining. Herring et al. at 474-476; Berry et al. at 181-183.

In addition, Katz et al., 1979 (Exhibit 7), detected retroviral RNA by *in vitro* translating the RNA in *Xenopus* oocytes and detecting the resultant proteins. Katz et al. at 448-449. Similarly, Philipson et al., 1978 (Exhibit 8), detected retroviral RNA by *in vitro* translating the RNA in reticulocyte extracts and detecting the resultant proteins. Philipson et al. at 197-198.

Moreover, Weber et al., 1974 (Exhibit 9), detected retroviral RNA by electron microscopy. Weber et al. at 1887-1889. Bender et al., 1976 (Exhibit 10), detected retroviral RNAs by electron microscopy using an SV40-Poly(dT) label. Bender et al. at 595-605. Castleman et al., 1980 (Exhibit 11), detected retroviral RNA by electron microscopy using anti-nucleotide antibodies as probes. Castleman et al. at 4487-4498.

Also, Pedersen et al., 1980 (Exhibit 12), detected retroviral RNA by digesting the RNA with RNase T<sub>1</sub> and labeling and detecting the resultant oligonucleotides.

Pedersen et al. at 350-364.

Due to the nature of applicants' claimed method, the method will work regardless of insertions, deletions, and variations in the HIV-1 sequence. In this way, the nature of applicants' claimed method provides a common attribute for the claim. Accordingly, applicants submit that the claimed invention is fully disclosed in the specification, and respectfully request withdrawal of the rejection.

Claim 47 was rejected under 35 U.S.C. § 112, first paragraph, as allegedly not reasonably providing enablement for detection of HIV-1 variants not disclosed in the specification. The Examiner concedes that the specification is enabled for detection of the specific HIV-1 sequence disclosed in the specification.

Applicants traverse the rejection. Once again, claim 47 is a claim for a method of detecting the presence of nucleic acid of a Human Immunodeficiency Virus Type 1 (HIV-1) in a biological sample. As shown by Exhibits 2-12, discussed above, many different well-known techniques could be used in the claimed method to detect HIV-1 nucleic acid. There is no reason to believe that the claimed method would not work with all HIV-1 species. Contrary to the Examiner's assertions, the claimed method does not require a specific sequence for detection, and will work regardless of insertions, deletions, and variations in the HIV-1 sequence. Consequently, applicants respectfully submit that Examiner's characterization as the process being unpredictable is in error. Accordingly, applicants submit that no undue experimentation would be required to practice the claimed invention, and respectfully request withdrawal of the rejection.



Applicants respectfully submit that this application is in condition for allowance.  
In the event that the Examiner disagrees, he is invited to call the undersigned to discuss  
any outstanding issues remaining in this application in order to expedite prosecution.

Please grant any extensions of time required to enter this response and charge  
any additional required fees to our deposit account 06-0916.

Respectfully submitted,

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# Isolation of a T-Lymphotropic Retrovirus from a Patient at Risk for Acquired Immune Deficiency Syndrome (AIDS)

**Abstract.** A retrovirus belonging to the family of recently discovered human T-cell leukemia viruses (HTLV), but clearly distinct from each previous isolate, has been isolated from a Caucasian patient with signs and symptoms that often precede the acquired immune deficiency syndrome (AIDS). This virus is a typical type-C RNA tumor virus, buds from the cell membrane, prefers magnesium for reverse transcriptase activity, and has an internal antigen (p25) similar to HTLV p24. Antibodies from serum of this patient react with proteins from viruses of the HTLV-I subgroup, but type-specific antisera to HTLV-I do not precipitate proteins of the new isolate. The virus from this patient has been transmitted into cord blood lymphocytes, and the virus produced by these cells is similar to the original isolate. From these studies it is concluded that this virus as well as the previous HTLV isolates belong to a general family of T-lymphotropic retroviruses that are horizontally transmitted in humans and may be involved in several pathological syndromes, including AIDS.

The acquired immune deficiency syndrome (AIDS) has recently been recognized in several countries (1). The disease has been reported mainly in homosexual males with multiple partners, and epidemiological studies suggest horizontal transmission by sexual routes (2) as well as by intravenous drug administration (3), and blood transfusion (4). The pronounced depression of cellular immunity that occurs in patients with AIDS and the quantitative modifications of subpopulations of their T lymphocytes (5) suggest that T cells or a subset of T cells might be a preferential target for the putative infectious agent. Alternatively, these modifications may result from subsequent infections. The depressed cellular immunity may result in serious opportunistic infections in AIDS patients, many of whom develop Kaposi's sarcoma (1). However, a picture of persistent multiple lymphadenopathies has also been described in homosexual males (6) and infants (7) who may or may not develop AIDS (8). The histological aspect of such lymph nodes is that of reactive hyperplasia. Such cases may correspond to an early or a milder form of the disease. We report here the isolation of a novel retrovirus from a lymph node of a homosexual patient with multiple lymphadenopathies. The virus appears to be a member of the human T-cell leukemia virus (HTLV) family (9).

The retrovirus was propagated in cultures of T lymphocytes from a healthy adult donor and from umbilical cord blood of newborn humans. Viral core proteins were not immunologically related to the p24 and p19 proteins of subgroup I of HTLV (9). However, serum of the patient reacted strongly with surface antigen (or antigens) present on HTLV-I-infected cells. Moreover, the ionic requirements of the viral reverse transcriptase were close to that of HTLV. Re-

cently, a type-C retrovirus was also identified in T cells from a patient with hairy cell leukemia. Analysis of the proteins of this virus showed they were related to, but clearly different from, proteins of previous HTLV isolates (10). Moreover, recent studies of the nucleic acid sequences of this new virus show it is less than 10 percent homologous to the earlier HTLV isolates (11). This virus was called HTLV-II to distinguish it from all the earlier, highly related viruses termed

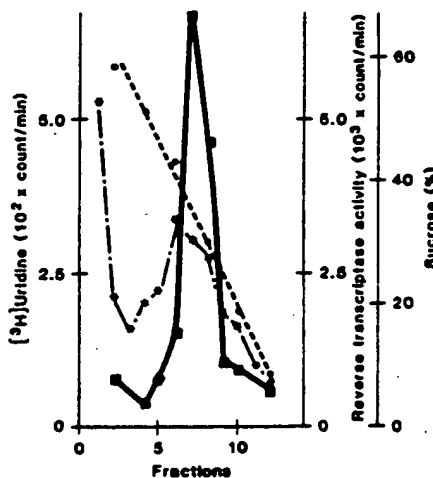


Fig. 1. Analysis of virus from patient 1 on sucrose gradients. Cord blood T lymphocytes infected with virus from patient 1 were labeled for 18 hours with [<sup>3</sup>H]uridine (28 Ci/mmole, Amersham; 20  $\mu$ Ci/ml). Cell-free supernatant was ultracentrifuged for 1 hour at 50,000 rev/min. The pellet was resuspended in 200  $\mu$ l of NTE buffer (10 mM Tris, pH 7.4, 100 mM NaCl, and 1 mM EDTA) and was centrifuged over a 3-ml linear sucrose gradient (10 to 60 percent) at 55,000 rev/min for 90 minutes in an IEC type SB 498 rotor. Fractions (200  $\mu$ l) were collected, and 30  $\mu$ l samples of each fraction were assayed for DNA polymerase activity with 5 mM Mg<sup>2+</sup> and poly(A) · oligo(dT)<sub>12-18</sub> as template primer; a 20- $\mu$ l portion of each fraction was precipitated with 10 percent trichloroacetic acid and then filtered on a 0.45- $\mu$ m Millipore filter. The <sup>3</sup>H-labeled acid precipitable material was measured in a Packard  $\beta$  counter.

cently, the new retrovirus reported here appears to also differ from HTLV-II. We tentatively conclude that this virus, as well as all previous HTLV isolates, belong to a family of T-lymphotropic retroviruses that are horizontally transmitted in humans and may be involved in several pathological syndromes, including AIDS.

The patient was a 33-year-old homosexual male who sought medical consultation in December 1982 for cervical lymphadenopathy and asthenia (patient 1). Examination showed axillary and inguinal lymphadenopathies. Neither fever nor recent loss of weight were noted. The patient had a history of several episodes of gonorrhea and had been treated for syphilis in September 1982. During interviews he indicated that he had had more than 50 sexual partners per year and had traveled to many countries, including North Africa, Greece, and India. His last trip to New York was in 1979.

Laboratory tests indicated positive serology (immunoglobulin G) for cytomegalovirus (CMV) and Epstein-Barr virus. Herpes simplex virus was detected in cells from his throat that were cultured on human and monkey cells. A biopsy of a cervical lymph node was performed. One sample served for histological examination, which revealed follicular hyperplasia without change of the general architecture of the lymph node. Immunohistological studies revealed, in paracortical areas, numerous T lymphocytes (OKT3<sup>+</sup>). Typing of the whole cellular suspension indicated that 62 percent of the cells were T lymphocytes (OKT3<sup>+</sup>), 44 percent were T-helper cells (OKT4<sup>+</sup>), and 16 percent were suppressor cells (OKT8<sup>+</sup>).

Cells of the same biopsied lymph node were put in culture medium with phytohemagglutinin (PHA), T-cell growth factor (TCGF), and antiserum to human  $\alpha$  interferon (12). The reason for using this antiserum was to neutralize endogenous interferon which is secreted by cells chronically infected by viruses, including retroviruses. In the mouse system, we had previously shown that antiserum to interferon could increase retrovirus production by a factor of 10 to 50 (13). After 3 days, the culture was continued in the same medium without PHA. Samples were regularly taken for assay of reverse transcriptase and for examination in the electron microscope.

After 15 days of culture, a reverse transcriptase activity was detected in the culture supernatant by using the ionic conditions described for HTLV-I (14). Virus production continued for 15 days

and decreased thereafter, in parallel with the decline of lymphocyte proliferation. Peripheral blood lymphocytes cultured in the same way were consistently negative for reverse transcriptase activity, even after 6 weeks. Cytomegalovirus could be detected, upon prolonged cocultivation with MRC5 cells, in the original biopsy tissue, but not in the cultured T lymphocytes at any time of the culture.

Virus transmission was attempted with the use of a culture of T lymphocytes established from an adult healthy donor of the Blood Transfusion Center at the Pasteur Institute. On day 3, half of the culture was cocultivated with lymphocytes from the biopsy after centrifugation of the mixed cell suspensions. Reverse transcriptase activity could be detected in the supernatant on day 15 of the coculture but was not detectable on days 5 and 10. The reverse transcriptase had the same characteristics as that released by the patient's cells and the amount released remained stable for 15 to 20 days. Cells of the uninfected culture of the donor lymphocytes did not release reverse transcriptase activity during this period or up to 6 weeks when the culture was discontinued.

The cell-free supernatant of the infected coculture was used to infect 3-day-old cultures of T lymphocytes from two umbilical cords, LC1 and LC5, in the presence of Polybrene (2  $\mu$ g/ml). After a lag period of 7 days, a relatively high titer of reverse transcriptase activity was detected in both of the cord lymphocyte cultures. Identical cultures, which had not been infected, remained negative. These two successive infections clearly show that the virus could be propagated on normal lymphocytes from either newborns or adults.

That this new isolate was a retrovirus was further indicated by its density in a sucrose gradient, which was 1.16, and by its labeling with [ $^3$ H]uridine (Fig. 1). Electron microscopy of the infected umbilical cord lymphocytes showed characteristic immature particles with dense crescent (C-type) budding at the plasma membrane (Fig. 2).

Virus-infected cells from the original biopsy as well as infected lymphocytes from the first and second viral passages were used to determine the optimal requirements for reverse transcriptase activity and the template specificity of the enzyme. The results were the same in all instances. The reverse transcriptase activity displayed a strong affinity for poly(adenylate · oligodeoxythymidylate) [poly(A) · oligo(dT)], and required  $Mg^{2+}$  with an optimal concentration (5 mM) slightly lower than that for HTLV

(14) and an optimal pH of 7.8. The reaction was not inhibited by actinomycin D. This character, as well as the preferential specificity for riboseadenylate · deoxythymidylate over deoxyadenylate · deoxythymidylate, distinguish the viral enzyme from DNA-dependent polymerases.

We then determined whether or not this isolate was indistinguishable from HTLV-I isolates. Human T-cell leukemia virus has been isolated from cultured T lymphocytes of patients with T lymphomas and T leukemias [for a review, see (9)]. The antibodies used were specific for the p19 and p24 core proteins of

HTLV-I. A monoclonal antibody to p19 (15) and a polyclonal goat antibody to p24 (16) were used in an indirect fluorescence assay against infected cells from the biopsy of patient 1 and lymphocytes obtained from a healthy donor and infected with the same virus. As shown in Table 1, the virus-producing cells did not react with either type of antibody, whereas two lines of cord lymphocytes chronically infected with HTLV (17) and used as controls showed strong surface fluorescence.

When serum from patient 1 was tested against infected lymphocytes from the biopsy the surface fluorescence was as

Table 1. Indirect immunofluorescence assay. Cells were washed with phosphate-buffered saline (PBS) and resuspended in the same buffer. Portions ( $5 \times 10^6$  cells) were spotted on slides, air-dried and fixed for 10 minutes at room temperature in acetone. Slides were stored at  $-80^\circ\text{C}$  until use. Twenty microliters of either monoclonal antibody to HTLV p19 (diluted 1/400 in PBS) or goat antibody to HTLV p24 (diluted 1/400 in PBS) or serum from patient 1 diluted 1/10 in PBS was applied to cells and incubated for 45 minutes at  $37^\circ\text{C}$ . The appropriate fluorescein-conjugated antiserum (antiserum to mouse, goat, or human immunoglobulin G) was diluted and applied to the fixed cells for 30 minutes at room temperature. Slides were then washed three times in PBS. Cells were stained with Evans blue solution for 15 minutes and then washed extensively with water before microscopic examination.

Cell type	Immunofluorescence (percent positive)		
	Antibody to p19	Antibody to p24	Serum from patient 1
Normal blood lymphocytes			
N 10916	—	—	—
LC <sub>1</sub>	—	—	—
HTLV-producing cells			
C <sub>91</sub> /PL	+ (90 to 100)	+ (90 to 100)	+ (90 to 100)
C <sub>10</sub> /MJ <sub>2</sub>	+ (90 to 100)	+ (90 to 100)	+ (90 to 100)
Virus-producing cells from			
Patient 1	—	—	+ (90 to 100)
LC <sub>1</sub> /patient 1	—	—	$\pm$ (0.5 to 2)
Patient 2	—	—	+ (90 to 100)



Fig. 2. Electron microscopy of thin sections of virus-producing cord lymphocytes. The inset shows various stages of particle budding at the cell surface.

intense as that of the control HTLV-producing lines. This suggests that serum of the patient contains antibodies that recognize a common antigen present on HTLV-I-producing cells and on the patient's lymphocytes. Similarly, cord lymphocytes infected with the virus from patient 1 did not react with antibodies to p19 or p24. Only a minor proportion of the cells (about 1 percent) reacted with the patient's serum. This may indicate that only this fraction of the cells was infected and produced virus. Alternatively, the antigen recognized by the patient's serum may contain cellular determinants that show less expression in T lymphocytes of newborns.

We also cultured T lymphocytes from a lymph node of another patient (patient 2) who presented with multiple adenopathies and had been in close contact with an AIDS case. These lymphocytes did not produce viral reverse transcriptase; however, they reacted in the immunofluorescence assay with serum from patient 1. Moreover, serum from patient 2 react-

ed strongly with control HTLV-producing lines (not shown). In order to determine which viral antigen was recognized by antibodies present in the two patients' sera, several immunoprecipitation experiments were carried out. Cord lymphocytes infected with virus from patient 1 and uninfected controls were labeled with [ $^{35}$ S]methionine for 20 hours. Cells were lysed with detergents, and a cytoplasmic S10 extract was made. Labeled virus released in the supernatant was banded in a sucrose gradient. Both materials were immunoprecipitated by antiserum to HTLV-I p24, by serum from patients 1 and 2, and by serum samples from healthy donors. Immunocomplexes were analyzed by polyacrylamide gel electrophoresis under denaturing conditions. Figure 3 shows that a p25 protein present in the virus-infected cells from patient 1 and in LC1 cells infected with this virus, was specifically recognized by serum from patients 1 and 2 but not by antiserum to HTLV-I p24 or serum of normal donors. Conversely, the p24

present in control HTLV-infected cell extracts was recognized by antibodies to HTLV but not by serum from patient 1. A weak band (lane 2, Fig. 3B) could hardly be seen with serum from patient 2, suggesting some similarities of the p25 protein from this patient's cells with HTLV-I p24. When purified, labeled virus from patient 1 was analyzed under similar conditions, three major proteins could be seen: the p25 protein and proteins with molecular weights of 80,000 and 45,000. The 45K protein may be due to contamination of the virus by cellular actin which was present in immunoprecipitates of all the cell extracts (Fig. 3).

These results, together with the immunofluorescence data, indicate that the retrovirus from patient 1 contains a major p25 protein, similar in size to that of HTLV-I but different immunologically. The DNA sequences of these and other members of the HTLV family are being compared. All attempts to infect other cells such as a B-lymphoblastoid cell line (Raji), immature or pre-T cell lines (CEM, HSB<sub>2</sub>), and normal fibroblasts (feline and mink lung cell lines) were unsuccessful.

The role of this virus in the etiology of AIDS remains to be determined. Patient 1 had circulating antibodies against the virus, and some of the latter persisted in lymphocytes of his lymph node (or nodes). The virus-producing lymphocytes seemed to have no increased growth potential in vitro compared to the uninfected cells. Therefore, the multiple lymphadenopathies may represent a host reaction against the persistent viral infection rather than hyperproliferation of virus-infected lymphocytes. Other factors, such as repeated infection by the same virus or other bacterial and viral agents may, in some patients, overload this early defense mechanism and bring about an irreversible depletion of T cells involved in cellular immunity.

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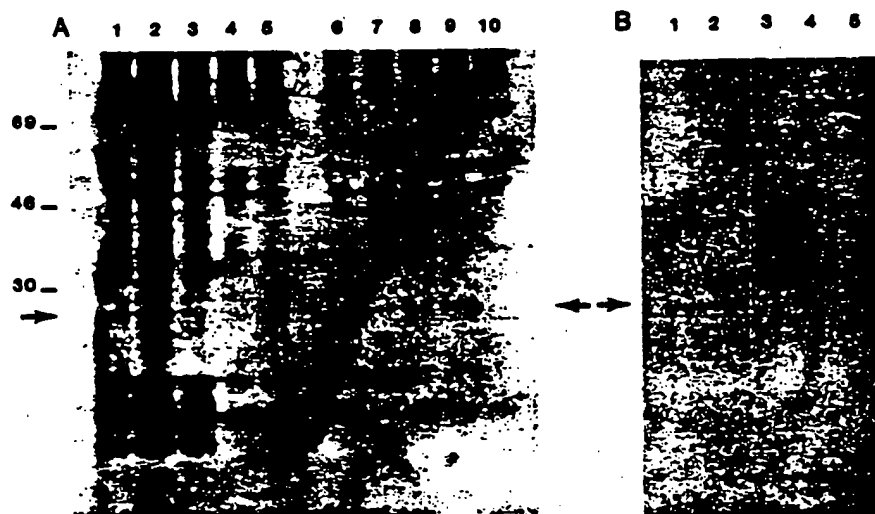


Fig. 3. Immunoprecipitation of  $^{35}$ S-labeled viral proteins. Cord blood T-lymphocytes infected with virus from patient 1 were incubated overnight in culture medium containing one-fifth of the normal concentrations of methionine in minimum essential medium, [ $^{35}$ S]methionine (1500 Ci/mmole, Amersham; 50  $\mu$ Ci/ml), and 10 percent dialyzed fetal calf serum. The virus was purified by banding on a sucrose gradient as described in Fig. 1. Labeled cells were resuspended in 10  $\mu$ l of saline and then lysed with 90  $\mu$ l of RIPA buffer (18) containing aprotinin (500 U/ml; Zymofren, Specia) at 4°C for 15 minutes. The supernatant of a 10,000g centrifugation of the cell extract was used for immunoprecipitation. A similar extract was made from HTLV-producing C<sub>91</sub>/PL cells (17). (A) Portions (20  $\mu$ l) of cell extracts were mixed with 6  $\mu$ l of serum, incubated for 2 hours at 37°C and overnight at +4°C. Then, 60  $\mu$ l of a suspension of Protein A-Sepharose (10 mg/ml in RIPA buffer) were added. After 45 minutes of incubation at 4°C, immunocomplexes bound to Protein A-Sepharose were washed five times with RIPA buffer by centrifugation, heated for 3 minutes at 100°C in denaturing buffer and electrophoresed on 12.5 percent polyacrylamide-SDS slab gel (19). Lanes 1 to 5: Extract of LC<sub>1</sub> cells infected with virus from patient 1 and tested against: 1, serum from patient 1; 2, serum from patient 2; 3, serum of a healthy donor; 4, goat antiserum to HTLV-Ip24; 5, normal goat serum. Lanes 6 to 10: C<sub>91</sub>/PL (HTLV-producing) cell extract tested with: 6, serum from patient 1; 7, serum from patient 2; 8, serum of a healthy donor; 9, goat antiserum to HTLV-Ip24; 10, normal goat serum. (B) Portions (20  $\mu$ l) of the band containing virus from patient 1 were treated with various antisera and processed as described for cell extracts. Lane 1, serum from patient 1; 2, serum from patient 2; 3, serum of a healthy donor; 4, serum of another healthy donor; 5, goat antiserum to HTLV-Ip24. Arrows indicate the p24-p25 protein. Molecular weights (in thousands) are indicated on the left.

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20. We thank Dr. Fradellizi for gifts of T-cell growth factor, R. C. Gallo for providing antibodies to HTLV and for HTLV-producing cells, Mrs. Le François for preparation of the cord lymphocytes, M. Laverne (Institut Pasteur, Production) for gifts of fluorescein-conjugated antisera, F. Huraud for performing some of the HTLV tests, and members of the French Working Group on AIDS for helpful discussion.

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## Detection of Nanogram Quantities of Retroviral RNA by Assay with Reverse Transcriptase

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A qualitative analytical method has been devised to locate nucleic acids in samples with amounts too small to detect by optical absorbance measurements. DNA polymerase (reverse transcriptase) from avian myeloblastosis virus is used to synthesize labeled DNA from a DNA or RNA template. The method can be used to detect DNA or RNA which has a primer or which has a poly(A) region to which oligo(dT) can be hybridized to form a primer. This method is particularly useful for locating high molecular weight RNA of retroviruses in gradient fractions in which as little as 0.7 ng of RNA can be detected. Since many RNAs, including mRNAs and bacteriophage RNAs, are templates for this enzyme, the method should have widespread applicability. DNAs which can serve as templates for reverse transcriptase can be distinguished from RNA by sensitivity to DNase, lack of sensitivity to RNase, and inhibition of the polymerase reaction by actinomycin D.

Retroviruses contain a high molecular weight (50–70S) RNA which serves as a template for synthesis of a complementary DNA (cDNA) transcript catalyzed by viral DNA polymerase (reverse transcriptase) (1,2). Many viruses are difficult to obtain in large amounts due to their poor growth in tissue culture. Frequently, however, small amounts of viral RNA, not detectable by measurements of optical absorbance, are adequate for experimental purposes. We have taken advantage of the fact that DNA polymerase from avian myeloblastosis virus (AMV) can utilize high molecular weight RNA from the viruses as template to synthesize cDNA in order to detect nanogram quantities of viral RNA in sedimentation velocity gradients.

### MATERIALS AND METHODS

#### Chemicals

[<sup>3</sup>H]TTP (specific activity 52 Ci/mmol) was purchased from New England Nuclear. Unlabeled nucleotides dATP, dGTP, and dCTP were obtained from P-L Biochemicals. Phenol was a product of Fisher Scientific, and *m*-cresol was an Aldrich Chemical product. Both were redis-

tilled before use. 8-Hydroxyquinoline was obtained from Fisher Scientific, and diethyl oxydiformalate was purchased from Eastman Kodak. Oligo(dT) was obtained from Collaborative Research.

All solutions, glassware, or other apparatus were either autoclaved or treated with 0.1% diethyl oxydiformalate in H<sub>2</sub>O and washed with diethyl oxydiformalate-treated H<sub>2</sub>O to eliminate possible ribonuclease.

#### Viruses and Reverse Transcriptase

AMV and purified AMV reverse transcriptase were provided by J. W. Beard, Life Sciences Research Labs through the NCI Office of Resources and Logistics of the Viral Oncology Program. The enzyme (150 µg of protein/ml) was diluted for use as 1 part enzyme to 99 parts 0.25 M Tris-HCl (pH 8.4), 0.4 M KCl, 0.075% Triton X-100, and 720 µM dithiothreitol. The precise concentrations of diluent are not critical. Commercial AMV reverse transcriptase (Schwarz/Mann) could also be used. AMV was received as frozen pellets. M-55 virus [isolated from NIH Swiss mouse uterus (10)], obtained as frozen culture fluid from P. T. Allen of our laboratory, was concentrated by centrifugation. Pellets of both viruses were resuspended in 5 mM Tris-HCl (pH 7.4, at 20°C), layered onto a 10 to 50% (w/w) sucrose gradient on a 60% (w/w) sucrose cushion, and centrifuged 17 hr at 17,000 rpm in a SW 27 rotor. The virus band, clearly visible by light scattering when illuminated by a high intensity lamp, was removed, diluted with 5 mM Tris-HCl (pH 7.4), and pelleted by centrifugation. Virus pellets were resuspended in 4 ml of 20 mM Tris-HCl (pH 9.0), 100 mM NaCl, and 1 mM EDTA; 0.25 ml of 20% sodium dodecyl sulfate was added, and the mixture was extracted with an equal volume of phenol-cresol-hydroxyquinoline (75/11/0.3, v/v/w) and an equal volume of chloroform. The aqueous layer was removed, and the interface and phenol layer were re-extracted with 0.5 vol of resuspension buffer (above). The combined aqueous layers were re-extracted twice with phenol-cresol-hydroxyquinoline, adjusted to 0.2 M LiCl, and the nucleic acids were precipitated by the addition of 2.5 vol of ethanol. After standing overnight at -20°C, the precipitate was pelleted, dried, and redissolved in 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM EDTA. The redissolved nucleic acid was layered onto a 10 to 30% (w/v) sucrose gradient in 5 mM Tris-HCl (pH 7.3) and 1 mM EDTA and centrifuged 5 hr at 40,000 rpm in an SW41 rotor along with a separate gradient containing [<sup>3</sup>H] RNA markers, 4S, 18S, and 28S (Schwarz/Mann). Fractions of 0.45 ml were collected in siliconized glass tubes. Fifty-microliter samples of each marker gradient fraction were counted in 5 ml of Aquasol. Ten-microliter portions of each fraction from the viral nucleic acid gradients were assayed with AMV reverse transcriptase in the presence of oligo(dT). The final concentrations of compo-

TABLE I  
CHARACTERIZATION OF VIRAL NUCLEIC ACIDS<sup>a</sup>

	10S	60S
Experiment 1		
No treatment	25,972 (100%)	5,099 (100%)
+RNase A (20 $\mu$ g/ml)	23,498 (90%)	155 (2%)
+DNase I (20 $\mu$ g/ml)	4,385 (17%)	6,133 (120%)
+Oligo(dT) (5 $\mu$ g/ml)	30,749 (118%)	17,017 (334%)
Experiment 2		
No treatment	90,145 (100%)	4,742 (100%)
+Oligo(dT) (5 $\mu$ g/ml)	88,849 (95%)	49,531 (1044%)
+Actinomycin D (50 $\mu$ g/ml)	15,114 (17%)	—
+Actinomycin D + oligo(dT)	17,225 (19%)	—
+RNase A	—	859 (18%)

<sup>a</sup> In Experiment 1, RNase A (Worthington Biochemicals, boiled to inactivate any contaminating activities), DNase I (Worthington Biochemicals, iodoacetate-treated to destroy any RNase activity), and oligo(dT) (Collaborative Research) were preincubated at 2.5 $\times$  the above final concentration 15 min at 37°C with 10  $\mu$ l of designated nucleic acid fraction before the reverse transcriptase reaction was begun. The counts indicated were obtained from a 40- $\mu$ l sample of the 50- $\mu$ l reaction volume. No preincubations were done in Experiment 2. Oligo(dT) was omitted from the assay mixture except where noted.

template activity of DNA was unaffected by the addition of oligo(dT), but that with RNA was boosted considerably by oligo(dT) (Table I). The degree of stimulation varied between three- and tenfold in different experiments. Stimulation by oligo(dT) depends upon the presence of poly(A) regions in the template such that oligo(dT) can hybridize to form a

TABLE 2  
SENSITIVITY OF THE DETECTION METHOD<sup>a</sup>

AMV RNA ( $\mu$ g/ml)	Counts per minute
34	35,608
3.4	7,534
0.34	1,219
0.07	202
0.034	128
0.007	43

<sup>a</sup> Oligo(dT)<sub>12-18</sub> was included at a final concentration of 5  $\mu$ g/ml. The no-RNA control value of 98 cpm was subtracted from each value. RNA concentration listed is that of the RNA solution tested. Ten microliters was used in our assays so that at 34  $\mu$ g/ml, for example, we included 0.34  $\mu$ g in the 50- $\mu$ l assay volume.

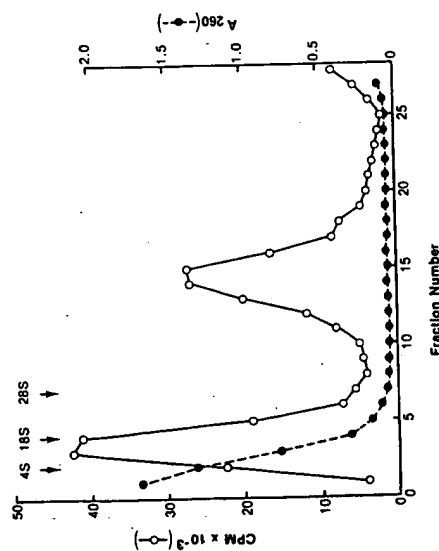


FIG. 1. Sedimentation velocity gradient of M-55 viral RNA. Sedimentation positions of RNA markers on a duplicate gradient are indicated by arrows. Ten-microliter portions of each fraction were assayed for viral RNA with AMV DNA polymerase.

nents of the assay mixture were as follows: 50 mM Tris-HCl (pH 8.3); 75 mM KCl; 0.015% Triton X-100; 10 mM dithiothreitol; 10 mM magnesium acetate; 5  $\mu$ g/ml of oligo(dT)<sub>12-18</sub>; 50  $\mu$ M each dATP, dCTP, and dGTP; 5  $\mu$ M [<sup>3</sup>H]TTP; 1.5  $\mu$ g of AMV polymerase/ml. Total reaction volume was 50  $\mu$ l. After incubation at 37°C for 1 hr, 40- $\mu$ l samples were pipetted onto Whatman 3MM filter paper disks, which were washed four times in acetic acid-HCl (5%/0.7%, v/v), twice in ethanol-ether (1/1, v/v), once in ether, and dried under a heat lamp, and precipitated radioactivity was determined by liquid scintillation counting.

## RESULTS AND DISCUSSION

The sedimentation velocity gradient profile of nucleic acids extracted from M-55 virus is shown in Fig. 1. Positions of RNA markers are indicated with arrows. As can be seen from the optical density profile, the nucleic acid peaks could not be detected by absorbance at 260 nm due to insufficient material. The RNA peak from M-55 virus appeared at approximately 60S. The earlier peak detected by enzyme assay at 10-12S is DNA. The DNA is contained within the virion, and similar DNAs are also found in NZB virus and other type C viruses. We have made a preliminary report of properties of this viral DNA (3), and further studies will be reported elsewhere. The DNA was an active template with AMV reverse transcriptase and illustrates that this analytical method can be of broader use than simply to detect high molecular weight viral RNA. The

free 3'-hydroxyl primer site (4). Whether or not other oligodeoxynucleotides would be effective depends upon the nucleotide sequence of the template. In one study (4) with globin mRNA as template, oligo(dC), oligo(dG), and oligo(dA) were reported to be ineffective as primers, while in a report using slime mold mRNA (5), oligo(dT) was most effective, but oligo(dC) and oligo(dG) also primed.

To determine the sensitivity of the method, we made a relatively large preparation of AMV high molecular weight RNA and assayed a series of RNA dilutions. The results are shown in Table 2. Under these conditions, we could reliably detect as little as 0.07 µg/ml of RNA or 0.7 ng of RNA in our assay. There are obviously a number of parameters affecting sensitivity, many dependent upon the nature and source of the RNA and DNA used. Several factors could be varied appropriately if increased sensitivity is necessary. Higher reverse transcriptase concentrations and longer incubation times as well as the use of additional labeled deoxynucleoside triphosphates should increase sensitivity. We have not explored oligo(dT) concentration. However, since DNA polymerases require 3'-hydroxyl groups for chain initiation, the number of oligo(dT) molecules hybridized to the template should be a significant factor in reaction rates. Variations in the percentage of the input oligo(dT) annealed to the template may explain the varied degree of stimulation we observed. Because of this variability the method may not be useful for quantitation. Our RNA dilution experiment did not give a linear response with dilution. As a qualitative tool, however, this method should have widespread applicability since a variety of RNAs from bacteriophage RNA (6) to slime mold mRNA (5) to mammalian mRNA (4,7) have been shown to be effective templates for AMV reverse transcriptase.

Weber *et al.* (8) noted the use of DNA polymerase from MC29 tumor virus to locate the sedimentation position of small quantities of DNA in CsCl density gradients. A more recent paper from the same laboratory (9) used AMV reverse transcriptase similarly. Their procedure was rather tedious because the DNA had to be removed from each fraction by ethanol precipitation to remove the inhibitory CsCl. In contrast, fractions from sucrose or glycerol gradients can be assayed directly. Nonetheless, the versatility of the method is illustrated.

The fact that oligo(dT) can raise the sensitivity of the assay many fold for some nucleic acids, particularly mRNA, and that AMV reverse transcriptase is commercially available should permit the application of this method to locate small amounts of RNAs previously undetectable.

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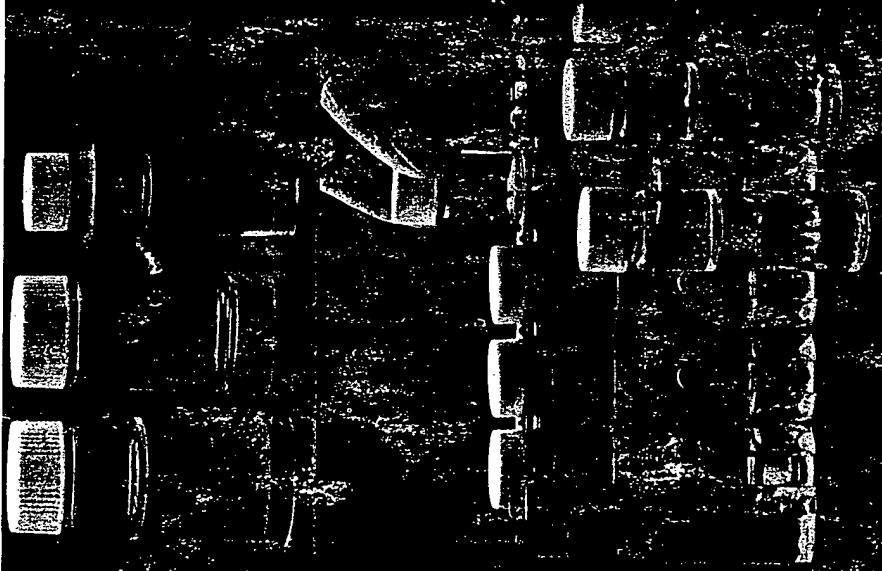
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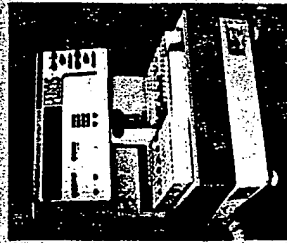


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# Detection, Sizing, and Quantitation of Polyadenylated Ribonucleic Acid in the Nanogram-Picogram Range<sup>†</sup>

Steven Tracy\* and David E. Kohne

**ABSTRACT:** A method is described for using very high specific activity [<sup>3</sup>H]poly(deoxythymidylate) [<sup>3</sup>H]poly(dT)] to detect, size, and quantitate subnanogram amounts of nonradioactive polyadenylated RNA. Short (~100 nucleotides long) [<sup>3</sup>H]poly(dT) is hybridized to the poly(adenylate) [poly(A)] tracts in polyadenylated RNAs. The RNA may then be sized and quantitated by sucrose gradient analysis. The addition of the small [<sup>3</sup>H]poly(dT) molecules does not significantly alter the *s* values of RNAs. The amount of [<sup>3</sup>H]poly(dT) hybridized to polyadenylated RNA increases linearly with the amount of RNA. A room temperature hydroxylapatite (HA) method has also been developed to detect and quantitate poly(A)-

containing RNA after hybridization to radioactive poly(dT). S-1 nuclease (S-1) analysis can also be used to measure the poly(A) content of polyadenylated RNA to less than nanogram RNA amounts. For both the S-1 and HA approaches, the amount of [<sup>3</sup>H]poly(dT) hybridized increases with the amount of RNA and the methods can detect to as little as 10<sup>-12</sup> g of polyadenylated RNA with [<sup>3</sup>H]poly(dT). Greater sensitivity is possible with higher specific activity poly(dT). The approaches presented here significantly extend the uses of radioactive homopolymers to detect, quantitate, and characterize RNAs containing complementary homopolymer tracts.

**P**oly(adenylate) [poly(A)]<sup>1</sup> tracts in RNA may be detected by utilizing the ability of poly(A) to hybridize with poly(uridylylate)[poly(U)] or poly(deoxythymidylate)[poly(dT)]. A number of approaches have been developed for the detection and quantitation of poly(A) which depend on annealing radioactive poly(U) or poly(dT) to the poly(A) tract (Gillespie et al., 1972; Fan & Baltimore, 1973; Kaufman & Gross, 1974; Sawin et al., 1977). The amount of poly(dT) or poly(U) hybridized to the RNA is then a measure of the poly(A) content of the RNA. A similar but slightly different poly(A) detection method employs the hybridization of oligo(deoxythymidylate)[oligo(dT)] to RNA fractions of a sucrose gradient. If poly(A) is present, an oligo(dT)-poly(A) complex is formed which may then be detected by monitoring cDNA synthesis by exogenously added reverse transcriptase (Strickland & Hellman, 1977). Another variation involves hybridizing iodinated poly(U) to RNA and then treating with bisulfite which deiodinates nonhybridized poly(U) but not poly(U) hybridized to poly(A) (Sawin et al., 1977). The above methods were also used to size the poly(A)-containing RNA by first sedimenting the RNA into a sucrose gradient and then detecting poly(A) in each gradient fraction by individually hybridizing aliquots of each fraction with radioactive poly(U) or poly(dT) (Rosbash & Ford, 1974; Sawin et al., 1977; Schlom et al., 1973). Radioactive homopolymers have also been used to detect homopolymer tracts present in higher organism DNAs (Shenkin & Burdon, 1972; Bishop et al., 1974).

The poly(A) detection sensitivity of the published methods ranges from 0.7 ng of AMV RNA detectable by a reverse transcriptase method (Strickland & Hellman, 1977) to 1-10 ng of various RNAs as assayed by hybridization with radioactive poly(U) or poly(dT) (Fan & Baltimore, 1973; Gillespie et al., 1972). None of the published methods demonstrated

a proportional linear relationship between the amount of poly(A) present and the amount of radioactivity detected as being associated with the poly(A).

The data presented here provide a solid base from which to use [<sup>3</sup>H]poly(dT) as a powerful tool for the detection, sizing, and quantitation of nanogram quantities of poly(A)-containing RNAs. Three methods [the sucrose gradient, HA, and S-1 nuclease (S-1) methods] are described which are useful for detecting and quantitating the reaction of [<sup>3</sup>H]poly(dT) with poly(A). Calibration curves constructed for each method are linear over at least 100-fold concentration range. Each method is useful for a different aspect of detecting poly(A). The poly(A) detection sensitivity of these methods is very high. By use of [<sup>3</sup>H]poly(dT) of 3.4 × 10<sup>8</sup> dpm per microgram specific radioactivity, the sensitivity of detection of poly(A)-containing RNA ranges from 1 to 50 pg depending on the RNA and the method of assay. The limit of detection of pure poly(A) is ~0.1 pg. The sucrose gradient assay for poly(A) has been greatly improved by hybridizing the [<sup>3</sup>H]poly(dT) to the RNA before sedimenting the RNA into a sucrose gradient. The gradient can then be fractionated directly into scintillation vials and assayed immediately for radioactivity. The [<sup>3</sup>H]poly(dT) attached to the RNA does not measurably alter the *s* value of the RNA. This method can be done with as little as 0.1-10 ng of RNA and greatly facilitates the characterization of extremely small amounts of poly(A)-containing RNAs.

## Materials and Methods

**Cells and Maintenance.** Moloney murine leukemia virus (M-MuLV) infected 3T3 cells, clone 1, were a gift from M. Goulian. Feline leukemia virus (FeLV) (Theilen strain) infected feline lymphoblastoid cells, termed FL-74 (Theilen et al., 1969), were obtained from T. Kawakami. 3T3 and FL-74 cells were grown as reported elsewhere (Van Beveren & Goulian, 1979; Theilen et al., 1969).

<sup>†</sup> From the Department of Biology, University of California, San Diego (S.T.), and the Center for Neurologic Study (D.E.K.), La Jolla, California 92093. Received December 28, 1979. This work was supported by the following National Institutes of Health awards: U.S. Public Health Service 5T 32GM07313 and CA 15376-03 and NO1-NS-8-2395.

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<sup>1</sup> Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; PB, phosphate buffer; NaDodSO<sub>4</sub>, sodium dodecyl sulfate; Sarcosyl, sodium *N*-lauroylsarcosinate; poly(dT), poly(deoxythymidylate); poly(U), poly(uridylylate); poly(A), poly(adenylate); poly(C), poly(cytidylylate); poly(G), poly(guanylylate); EDTA, ethylenediaminetetraacetic acid; HA, hydroxylapatite.

**Isolation and Purification of RNA.** Murine embryonic  $\alpha$ -fetoprotein ( $\alpha$ -FP) mRNA was the gift of T. Tamaoki, Semliki Forest Virus (SFV) [ $^3$ H]RNA was the gift of I. Kennedy, and encephalomyocarditis (EMC) virus RNA was the gift of D. Rowlands. Polio virus was grown and RNA isolated as described (Kacian & Myers, 1976). Avian myeloblastosis virus (AMV) was isolated from the serum of infected chickens as described by Myers et al. (1977).

Poly(A)-containing viral RNA was isolated from disrupted virus on oligo(dT)-cellulose (OTC) (Collaborative Research; T-3) by a modification of the method of Smith et al. (1976). Concentrated virus in 0.1 M NaCl, 10 mM Tris, pH 7.5, and 1 mM EDTA (TNE) was lysed on ice with 1% Sarcosyl (K and K Laboratories) in the presence of 200  $\mu$ g/mL proteinase K (E. Merck) for 15 min. The lysate was transferred to 37  $^{\circ}$ C for 15 min and then cooled to room temperature. The lysate was made 6 M in NaSCN from a stock of 8 M NaSCN, shaken gently at room temperature for 5 min, and diluted to 1 M NaSCN with OTCB (0.5 M NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, and 1% Sarcosyl). The lysate was then dripped through a 3-mL bed of OTC in a sterile plastic pipet at a flow rate of 1 mL/min. The column was then washed with 30 mL of each of the following buffers: (1) 1 M NaSCN and OTCB; (2) 4 M NaCl and 10 mM Tris, pH 7.5; (3) OTCB; (4) OTCB without Sarcosyl. Poly(A)-containing RNA was eluted with sterile glass-distilled water in 0.5-mL aliquots. The concentration of the RNA was calculated from the absorbance at 260 nm (25 units = 1 mg of RNA). Nonradioactive RNA was aliquoted and frozen at  $-70^{\circ}$ C. Radioactive RNA was aliquoted and stored at  $4^{\circ}$ C. RNA isolated in this manner routinely had an  $A_{260}/A_{280}$  absorbance ratio greater than 2.0.

**Reverse Transcriptase Synthesis of [ $^3$ H]Poly(dT).** AMV reverse transcriptase was supplied from the Logistics Program of NCI. [ $^3$ H]Poly(dT) was synthesized in a 50- $\mu$ L reaction mix in 0.4-mL Eppendorf conical polypropylene tubes. Reagents were added in the following order at room temperature, with the exception of reverse transcriptase: 29.75  $\mu$ L of water, 2  $\mu$ L of 0.1 M sodium pyrophosphate, 4  $\mu$ L of 1 mM [ $^3$ H]dTTP (50 Ci/mmol, 50 mCi/mL) (New England Nuclear), 2  $\mu$ L of 1 M KCl, 4  $\mu$ L of 0.1 M MgCl<sub>2</sub>, 2.5  $\mu$ L of 1 M Tris, pH 8.3, 2.5  $\mu$ L of 0.1 M dithiothreitol, 1  $\mu$ L of 0.5% Nonidet P-40, 1.25  $\mu$ L of 2 mg/mL poly(A)-oligo-(dT)<sub>12-18</sub> (Miles), and 1  $\mu$ L (6127 units/mL) of reverse transcriptase. The reaction mixture was then briefly vortexed to mix and placed at  $40^{\circ}$ C for 20 min. The reaction was stopped with 100  $\mu$ L of 0.1 M EDTA, pH 7.5, and 2  $\mu$ L of 20% NaDodSO<sub>4</sub> and shaken once with 10:90 phenol-chloroform. The aqueous layer was put over a 10-mL bed of Sephadex G-100 (Pharmacia) in TNE and 0.1% Sarcosyl. The excluded peak was made 30  $\mu$ g with purified glycogen and 0.5 M in NaCl and precipitated in ethanol overnight at  $-20^{\circ}$ C. The precipitate was collected by centrifugation, suspended in 200  $\mu$ L of water, made 0.4 M in NaOH, and incubated at  $60^{\circ}$ C, 1 h. This mixture was neutralized by passage over a new Sephadex G-100 column as above. The eluting poly(dT) in the excluded volume was again ethanol precipitated, collected by centrifugation, and resuspended in a convenient volume of 0.01 M each of NaCl, Tris, pH 7.5, and EDTA, pH 7.5. The poly(dT) was stored at  $4^{\circ}$ C.

The average length of the [ $^3$ H]poly(dT) as measured by denaturing gel electrophoresis was 150–170 bases. After 12 months of storage, the average length was 70 bases long (data not shown).

**Hybridization Conditions.** Samples of RNA for hybridization with [ $^3$ H]poly(dT) were standardly annealed in 0.5 M

NaCl and 0.03% Sarcosyl at room temperature in 10–20  $\mu$ L. Sarcosyl or NaDodSO<sub>4</sub> (0.01–0.03%) was found to inhibit nonspecific sticking of the poly(dT) to surfaces. MS-2 bacteriophage RNA does not anneal to poly(dT) (Gillespie et al., 1972) and may be used as a carrier if desired. Unless noted, [ $^3$ H]poly(dT) was present in the reactions in at least a five- to sixfold excess relative to the expected amount of poly(A) in the RNA. The measured  $C_0t_{1/2}$  of the [ $^3$ H]poly(dT)-poly(A) reaction under these conditions was  $\sim 1 \times 10^{-6}$  mol s L<sup>-1</sup>. Hybridization times were typically 30 min unless otherwise specified. For layering on sucrose gradients, reactions were then made up to 100  $\mu$ L with 0.5 M NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, and 0.03% Sarcosyl. Reactions were diluted to 2 mL with 0.1 M PB and 0.02% NaDodSO<sub>4</sub> for hydroxylapatite (HA) analysis. For S-1 nuclease analysis, the reactions were diluted to 1 mL with 0.3 M NaCl, 0.05 M NaOAc, pH 4.5, and 0.003 M ZnSO<sub>4</sub>.

**Sucrose Gradient Analysis.** Sucrose solutions were in 0.1 M NaCl, 10 mM Tris, pH 7.5, and 1 mM EDTA. Linear 5–20% (w/v) gradients were poured and centrifuged in a Beckman SW50.1 rotor, 45000 rpm,  $20^{\circ}$ C, for various times depending on the size of the RNA. Gradients were stopped without breaking to minimize mixing. Sucrose was RNase free (Schwarz/Mann), and all stock buffers were autoclaved prior to use. Gradients were fractionated from below directly into scintillation vials. To each vial was added 1 mL of water and 6 mL of Aquasol-2 (New England Nuclear) prior to counting in a liquid scintillation counter. For purposes of quantitation, a peak is defined by all material between the lowest levels of [ $^3$ H]poly(dT) on either side of the peak.

**Hydroxylapatite Analysis.** RNA and [ $^3$ H]poly(dT) were hybridized at room temperature in 10–30  $\mu$ L of 0.5 M NaCl or 1 M NaCl and 0.02% NaDodSO<sub>4</sub> for varying times. The reaction mixture was diluted with 1 mL of 0.1 M PB (phosphate buffer composed of equimolar amounts of NaH<sub>2</sub>PO<sub>4</sub> and Na<sub>2</sub>HPO<sub>4</sub>) and 0.02% NaDodSO<sub>4</sub>. The sample was passed rapidly over a bed of 0.5–1 mL of HA (HTP DNA Grade, Bio-Rad) equilibrated with 0.1 M PB and 0.02% NaDodSO<sub>4</sub> at room temperature. A 3-mL wash of the same buffer eluted the unbound [ $^3$ H]poly(dT). Bound poly(dT)-RNA complexes were eluted with 3-mL washes of 0.3 M PB. The HA was then dissolved in 2 mL of 6 N HCl, and all the fractions were assayed for radioactivity.

**S-1 Nuclease Analysis.** Reaction mixtures were diluted to 1 mL with S-1 nuclease buffer consisting of 0.3 mL of NaCl, 0.003 M ZnSO<sub>4</sub>, and 0.05 M NaOAc, pH 4.5. To this was added 2  $\mu$ g of *Escherichia coli* DNA and 50 units of S-1 nuclease (Calbiochem). The mixture was incubated at room temperature ( $20$ – $24^{\circ}$ C) for 1 h and then assayed for acid-precipitable radioactivity. Both the acid-precipitable and acid-soluble fractions were collected and assayed for radioactivity.

**Analysis of Hybridization Results.** All quantitations of poly(A) results have been corrected for the self-reactive fraction of [ $^3$ H]poly(dT); the data presented have had the self-reactive fraction subtracted. One milligram of poly(A) represented 30  $A_{260}$  units, and 1 mg of biological RNA represented 25  $A_{260}$  units. The specific radioactivity of the [ $^3$ H]poly(dT) was  $3.4 \times 10^8$  dpm/ $\mu$ g. Data presented as counts per minute represent a tritium counting efficiency of 36%.

## Results

**Conditions for Hybridizing [ $^3$ H]Poly(dT) to Poly(A) RNA.** The salt concentration used for these reactions, 0.5 M NaCl, yielded close to optimum rates of hybridization at room tem-

Table I: Rates of Hybridization of [<sup>3</sup>H] Poly(dT) with Poly(A)

	[poly(dT)]/[ poly(A)]	[NaCl] (M)	$C_{0t_{1/2}}$ (mol s L <sup>-1</sup> ) (based on excess nucleic acid)
excess poly(A) + [ <sup>3</sup> H] poly(dT)	0.059	0.1	$1 \times 10^{-5}$
	0.059	0.5	$6 \times 10^{-7}$
	0.059	1	$2 \times 10^{-7}$
excess [ <sup>3</sup> H] poly(dT) + 28 nucleotide long poly(A)	9	0.5	$8 \times 10^{-7}$
excess [ <sup>3</sup> H] poly(dT) + poly(A) greater than 280 nucleotides	9	0.5	$1 \times 10^{-6}$
excess poly(dT) + polio RNA	10 <sup>a</sup>	0.5	$1 \times 10^{-6}$

<sup>a</sup> Poly(dT) excess was calculated over the amount of poly(A) in polio RNA. Hybridizations were performed and analyzed as described under Materials and Methods.

Table II: Characterization of [<sup>3</sup>H] Poly(dT)<sup>a</sup>

[ <sup>3</sup> H]- poly(dT) prepn	assay method	% reaction with excess poly(A)	% reaction with an ex- cess of other RNAs	% reaction when no RNA is added
1	HA	84		
1	HA		polio 84	
1	HA		MS-2 2.4	
1	HA			2.3
2	HA	91.6		
2	HA			3.6
3	HA	98		
3	HA			1.3
3	S-1	92		
3	S-1			2.3

<sup>a</sup> Newly made preparations 1, 2, and 3 of [<sup>3</sup>H] poly(dT) were sized respectively at 4 S in alkaline sucrose. Reactions were assayed by HA or S-1 nuclease as described under Materials and Methods. [<sup>3</sup>H] Poly(dT) preparation 3 was self-reacted and passed twice over HA in 0.1 M PB at room temperature in order to reduce the amount of apparent self-reaction. Before passage over the HA, 6–7% self-reaction was observed.

perature as measured by hydroxylapatite. Data regarding rates of hybridization of poly(dT) to poly(A) are summarized in Table I.

The [<sup>3</sup>H] poly(dT) is used to detect the presence of a small amount of poly(A) attached to a large complex RNA molecule. The kinetics of reaction of [<sup>3</sup>H] poly(dT) with the poly(A) of such an RNA molecule was examined by measuring the rate of hybridization of [<sup>3</sup>H] poly(dT) with polio type 1 RNA under conditions of a 10-fold excess of [<sup>3</sup>H] poly(dT) relative to the amount of poly(A) present in the polio virus RNA. Polio virus contains ~0.6–1% poly(A). This is equivalent to a sequence consisting of ~50–85 adenine residues/average molecule (Yogo & Wimmer, 1972; Hraby & Roberts, 1976). In 0.5 M NaCl, the [<sup>3</sup>H] poly(dT)  $C_{0t_{1/2}}$  [based on poly(A) content] of this reaction is  $\sim 1 \times 10^{-6}$  mol s L<sup>-1</sup> or about the same as that seen for excess poly(dT) and poly(A) (Table I).

**Characterization of [<sup>3</sup>H] Poly(dT).** [<sup>3</sup>H] Poly(dT) produced as described under Material and Methods was examined for its ability to react with poly(A), its ability to react with non-poly(A)-containing RNA, its self-hybridization characteristics, its ability to bind hydroxylapatite immediately after being denatured, and its size by alkaline sucrose sedimentation analysis. Table II summarizes these data. Different [<sup>3</sup>H]-poly(dT) preparations self-reacted to an extent of 2–3% and

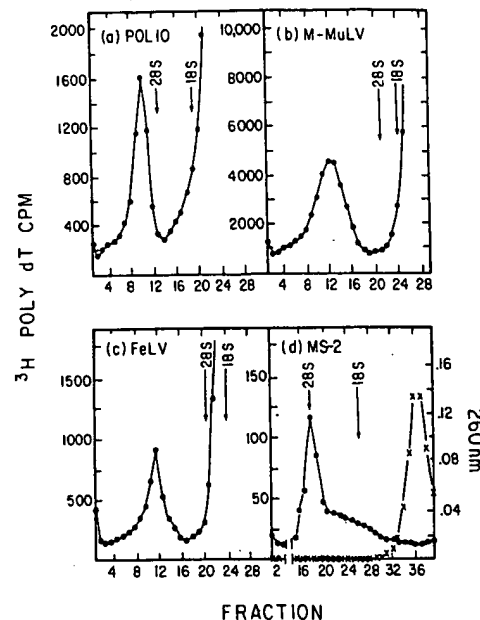


FIGURE 1: Detection of different RNAs with [<sup>3</sup>H] poly(dT). (a) 6 ng of polio virus RNA was annealed with 1.73 ng of [<sup>3</sup>H] poly(dT) in 20  $\mu$ L of 0.5 M HCl and 0.03% Sarcosyl at room temperature, 20 min. The reaction mixture was made 100  $\mu$ L with 0.5 M NaCl, 0.03% Sarcosyl, 10 mM Tris, pH 7.5, and 1 mM EDTA and layered onto a 5–20% (w/v) linear sucrose gradient made in 0.1 M NaCl, 10 mM Tris, pH 7.5, and 1 mM EDTA. Centrifugation was in a Beckman L5-50 ultracentrifuge with the Beckman SW50.1 rotor: 45 000, 100 min, 20 °C, slow acceleration, and no brake. Gradients were fractionated from below directly into scintillation vials to which were added 1 mL of water and 6 mL of ACS (Fisher). Vials were counted in a liquid scintillation counter. (b) 10.5 ng of M-MuLV RNA was annealed with 1.73 ng of [<sup>3</sup>H] poly(dT), centrifuged, and assayed as in (a) except that centrifugation was for 45 min. (c) 1.9 ng of FeLV RNA was annealed with 1.73 ng of [<sup>3</sup>H] poly(dT) and treated as in (b). (d) 22  $\mu$ g of MS-2 RNA was hybridized with 0.35 ng of [<sup>3</sup>H] poly(dT) in 100  $\mu$ L of 0.5 M NaCl and 0.03 Sarcosyl for 20 min at room temperature. The reaction mixture was centrifuged as in (a). The gradient was fractionated from below, 1 mL of water was added to each fraction, and the absorbance at 260 nm was read for the fractions. 6 mL of Beta Phase (WestChem Chemical Co.) was then added to each sample, and the vials were counted in a liquid scintillation counter: (●)  $A_{260}$ ; (×) [<sup>3</sup>H] poly(dT) cpm.

reacted to greater than 80% with an excess of poly(A) and polio RNA. No significant reaction of the [<sup>3</sup>H] poly(dT) with an excess of RNA from the bacteriophage MS-2 could be detected.

**Sizing of Poly(A)-Containing RNA: Sucrose Gradient Analysis.** The hybridization of [<sup>3</sup>H] poly(dT) with the poly(A) portion of nonradioactive RNA permits the detection of nanogram amounts of these RNAs. The size of these poly(A)-containing RNAs labeled with [<sup>3</sup>H] poly(dT) can be determined by sucrose gradient analysis. Figure 1 shows the result of one typical experiment detecting poly(A)-containing RNA with [<sup>3</sup>H] poly(dT). Polio virus RNA, FeLV RNA, and M-MuLV RNA were hybridized to [<sup>3</sup>H] poly(dT) in separate reactions and layered onto 5–20% sucrose gradients. Each RNA sedimented at its expected  $s$  value. Less than 20 ng of RNA was used for each of these experiments, an amount undetectable by standard spectrophotometric means. More than 20  $\mu$ g of MS-2 bacteriophage RNA which contains no poly(A) was also reacted with [<sup>3</sup>H] poly(dT) and analyzed both spectrophotometrically and as described above. The [<sup>3</sup>H]-poly(dT) did not exhibit any binding to the MS-2 RNA which was detected in the gradient by its absorbance at 260 nm.

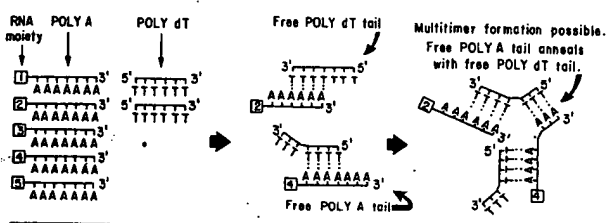
It was expected that the amount of [<sup>3</sup>H] poly(dT) actually hybridized to the poly(A)-containing RNA would not be

Table III: Comparison of [ $^3\text{H}$ ]Poly(dT)-Determined and Published  $s$  Values of RNAs<sup>a</sup>

RNA	reported value (S)	[ $^3\text{H}$ ]-poly(dT)-determined value (S)	internally labeled value (S)
M-MuLV	60-70 <sup>b</sup>	60	60
FeLV	58 <sup>c</sup>	58-60	58-60
AMV	65 <sup>d</sup>	58-63	ND <sup>e</sup>
SFV	45 <sup>f</sup>	46	46
polio	35 <sup>g</sup>	33-34	ND
$\alpha$ -FP mRNA	18 <sup>h</sup>	18	ND

<sup>a</sup> [ $^3\text{H}$ ]Poly(dT)-determined and internally labeled  $s$  values are relative to 18 and 28S rRNA markers. <sup>b</sup> Lai & Duesberg (1972). <sup>c</sup> Brian et al. (1975). <sup>d</sup> Erickson (1969). <sup>e</sup> ND = not determined. <sup>f</sup> Sonnabend et al. (1967). <sup>g</sup> Bishop et al. (1965). <sup>h</sup> Tamaoki et al. (1976).

## (a) [POLY A] &gt; [POLY dT]



## (b) [POLY A] &lt; [POLY dT]

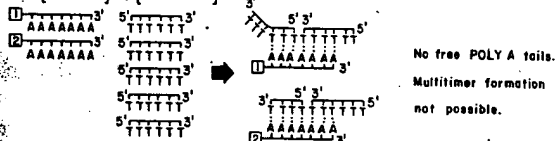
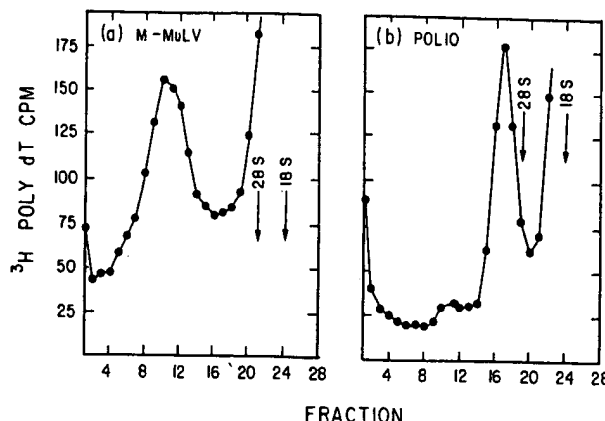


FIGURE 2: Hypothetical interaction of poly(A) and poly(dT). Hybridization of poly(A) with poly(dT) in (a) poly(A) excess and (b) poly(dT) excess.

enough to markedly change the  $s$  value of the RNA. Smaller RNAs would be expected to reflect more change in  $s$  value. The smallest poly(A)-containing RNA checked was murine  $\alpha$ -fetoprotein ( $\alpha$ -FP) mRNA. When newly isolated, this RNA was sized at 18 S by polyacrylamide gel electrophoresis (Tamaoki et al., 1976). This  $\alpha$ -FP mRNA also contains the largest fraction of poly(A),  $\sim 5\%$ . This mRNA was repeatedly sized at 18-18.5 S with [ $^3\text{H}$ ]poly(dT), indicating that saturation of as much as 5% of the total molecule with [ $^3\text{H}$ ]poly(dT) does not appreciably change the  $s$  value. A summary of [ $^3\text{H}$ ]poly(dT)-determined  $s$  values for a variety of poly(A)-containing RNAs is presented in Table III. These data show a good correlation between [ $^3\text{H}$ ]poly(dT)-determined sizes and those reported by others using conventional methods.

When using the [ $^3\text{H}$ ]poly(dT) method to size and quantitate poly(A)-containing RNA, it is important to hybridize the RNA to an excess of [ $^3\text{H}$ ]poly(dT), relative to the amount of poly(A) present in the RNA. Good results are obtained with a 6-10-fold excess of poly(dT). When the amount of polio poly(A) was in excess of the [ $^3\text{H}$ ]poly(dT), some of the polio RNA sedimented more rapidly than the main peak. Most of the polio RNA is present in the main monomer peak which has the  $s$  value expected for intact polio RNA. However, additional peaks which appear to be dimers and trimers of polio RNA are also seen (data not shown).

The reason for these multimer peaks may be explained by a scheme such as that presented in Figure 2a. This figure portrays a case of poly(A) excess relative to poly(dT). In this case, poly(dT) does not saturate all the poly(A) available and,

FIGURE 3: Sedimentation profiles of subnanogram amounts of [ $^3\text{H}$ ]poly(dT)-labeled RNAs. (a) 0.3 ng of M-MuLV RNA were annealed to 1.73 ng of [ $^3\text{H}$ ]poly(dT), sedimented, and assayed as described for Figure 1b. (b) 0.5 ng of polio virus RNA was annealed to 0.26 ng of [ $^3\text{H}$ ]poly(dT) in 20  $\mu\text{L}$  of 0.5 M NaCl at room temperature, 30 min and then layered onto a 5-20% (w/v) linear sucrose gradient and centrifuged in the SW50.1 rotor: 45 000 rpm, 85 min, and 5  $^{\circ}\text{C}$ . The gradient was assayed as in Figure 1a.

consequently, poly(A) tails may exist. These may hybridize with poly(dT) already partially hybridized to the poly(A) region of a different molecule of RNA, with the result that a molecule of poly(dT) links two or more RNA molecules to form multimers. Poly(dT) at an excess will completely saturate available poly(A), leaving no free, unhybridized poly(A) tails. Figure 2b presents a schematic view of the poly(dT) excess situation. Sucrose gradient analysis at high salt concentration and at low temperature may also encourage the formation of multimer peaks and also enhance the aggregation of RNA (Parrish, 1972).

The [ $^3\text{H}$ ]poly(dT) can be used to detect subnanogram quantities of poly(A)-containing RNA. Figure 3 presents the sedimentation profiles obtained by using 0.3 ng of M-MuLV RNA and 0.5 ng of polio RNA. The high molecular weight RNA peaks are readily apparent. The polio RNA peak contains  $\sim 500$ -600 cpm while the M-MuLV peak has  $\sim 1000$  cpm. The peak regions could be further separated from the nonhybridized [ $^3\text{H}$ ]poly(dT) by using a longer gradient.

It is at times useful to eliminate the nonhybridized [ $^3\text{H}$ ]poly(dT) before analyzing the sample on a sucrose gradient. This can be accomplished by first passing the sample over HA (Materials and Methods) in 0.1 M PB and 0.02% NaDodSO<sub>4</sub>. Under these conditions, the single-strand [ $^3\text{H}$ ]poly(dT) will pass through the column while the [ $^3\text{H}$ ]poly(dT) hybridized to the RNA will adsorb to the HA. The adsorbed fraction can be recovered by high salt elution and analyzed on a sucrose gradient.

[ $^3\text{H}$ ]Poly(dT) may also be used to detect denatured RNA. Glyoxal denatures RNA and DNA by forming adducts with the guanine residues, preventing G-C base pair formation and thereby effectively denaturing the molecules (McMaster & Carmichael, 1977). [ $^3\text{H}$ ]Poly(dT) may be used in conjunction with this method to label denatured polyadenylated RNA because glyoxylation does not affect the poly(A) sequence of the RNA (data not shown).

Breakdown of a particular RNA preparation may also be rapidly assessed by using [ $^3\text{H}$ ]poly(dT) by observing the profile of radioactivity on a sucrose gradient. One preparation of polio RNA which yielded 18S (alkaline) cDNA when transcribed with reverse transcriptase using oligo(dT)<sub>12-18</sub> as the primer (Kacian & Myers, 1976) was shown to be 34 S in sucrose by using [ $^3\text{H}$ ]poly(dT) (data not shown). A different preparation



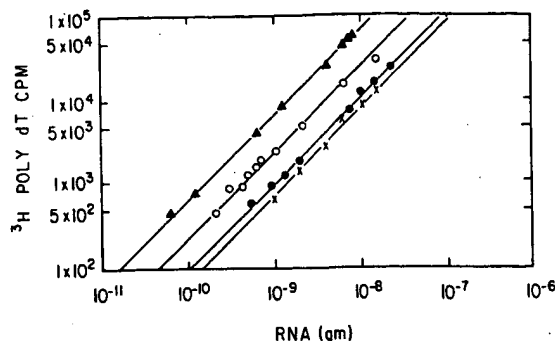


FIGURE 4: Standard curves for quantitation of specific  $[^3\text{H}]$ poly(dT)-labeled RNAs. The amount of  $[^3\text{H}]$ poly(dT) radioactivity in the gradient peak is plotted vs. the amount of RNA in the specific reaction: ( $\Delta$ )  $\alpha$ -FP mRNA; (O) M-MuLV RNA; ( $\bullet$ ) FeLV RNA; ( $\times$ ) polio virus RNA.

which yielded 4-5S cDNA when transcribed in the same manner by reverse transcriptase showed no detectable RNA larger than the  $[^3\text{H}]$ poly(dT) itself. This RNA was not heated before reacting with the  $[^3\text{H}]$ poly(dT). The absence of any peak indicated that the RNA was badly degraded.

The poly(dT) method may also be used to determine whether a poly(A)-containing RNA is completely intact; that is, none of its phosphodiester bonds have been broken. A situation often occurs where only a few random nicks (i.e., breaking of phosphodiester bonds) of the RNA have occurred. Unless denatured, this RNA will remain intact and will give the proper  $s$  value when sized with  $[^3\text{H}]$ poly(dT). The nicks can be detected, however, by first denaturing the RNA and then reacting it with the  $[^3\text{H}]$ poly(dT) and sizing it. The resulting sedimentation profile will show RNA with a lower  $s$  value than expected.

**Quantitation of RNA Containing Poly(A) by  $[^3\text{H}]$ Poly(dT) Hybridization.** If the proper calibration curves are available, the amount of  $[^3\text{H}]$ poly(dT) present in a particular sucrose gradient peak can serve as a measure of the quantity of total RNA present in that peak. Construction of such a calibration curve involves determining how much  $[^3\text{H}]$ poly(dT) will hybridize with differing amounts of a particular poly(A)-containing RNA. Figure 4 presents such a calibration curve for a variety of poly(A)-containing RNAs. Varying amounts of each RNA were hybridized to an excess of  $[^3\text{H}]$ poly(dT) relative to the poly(A) present in the RNA. The reaction mixture was then subjected to sucrose gradient analysis, and the amount of  $[^3\text{H}]$ poly(dT) (counts per minute) in each peak was determined. The radioactivity in the peaks was plotted against the respective RNA concentrations. The results of such calibrations are depicted in Figure 4.

It is apparent that there is an essentially linear relationship between the amount of RNA in the reaction and the amount of  $[^3\text{H}]$ poly(dT) hybridized to it. This relationship remains linear over a range where amounts of RNA differ by 100-fold. It is also clear that subnanogram amounts of poly(A)-containing RNA can be quantitatively assayed by this method (see also Figure 3). The results presented in Figure 4 show an internal consistency which speaks well for the accuracy and reproducibility of the method. The slope of each of the lines is equal to  $\sim 1.02$ . This indicates that an increase in the quantity of a specific poly(A)-containing RNA will result in an essentially proportional increase in the quantity of  $[^3\text{H}]$ poly(dT) hybridized to the RNA.

**Hydroxylapatite and S-1 Nuclease Methods of Quantitating RNA Containing Poly(A).** The hydroxylapatite (HA) and S-1 methods of detecting poly(A)- $[^3\text{H}]$ poly(dT) hybrid-

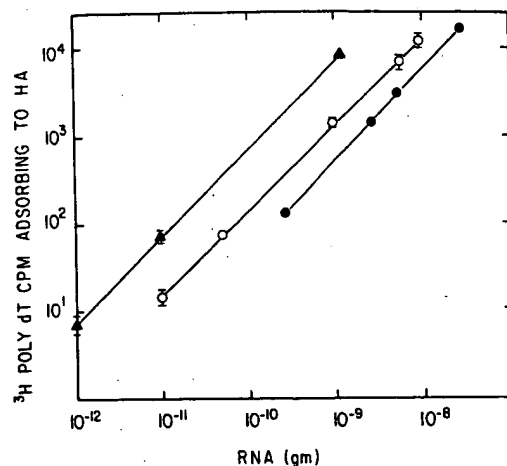


FIGURE 5: HA and S-1 nuclease standard curves for quantitation of polio and  $\alpha$ -FP RNAs. The cpm at each point has been corrected for the amount of self-reaction of the  $[^3\text{H}]$ poly(dT). Assays were performed as described under Materials and Methods: ( $\Delta$ )  $\alpha$ -FP mRNA assayed by HA; (O) polio RNA assayed by HA; ( $\bullet$ ) polio RNA assayed by S-1 nuclease.

ization add a useful dimension to the quantitation of poly(A)-containing RNAs. These methods can be carried out more rapidly than sucrose gradients and have a much greater potential sensitivity.

Figure 5 shows a calibration curve constructed by hybridizing different amounts of  $\alpha$ -FP and polio RNA to excess  $[^3\text{H}]$ poly(dT) and then analyzing the reaction mixtures by the HA method. The amount of  $[^3\text{H}]$ poly(dT) adsorbed to the HA is then plotted vs. the respective RNA concentration. These data show that there is an essentially linear relationship between the quantity of RNA and the amount of radioactive poly(dT) adsorbing to the HA. This linearity extends over a range where amounts of RNA differ by 1000-fold. The slope of the polio and  $\alpha$ -FP lines is 0.98. These slopes indicate that an increase in the quantity of RNA will result in a proportional increase in the amount of radioactivity hybridized to the RNA.

It is apparent that the HA method is much more sensitive in detecting small amounts of poly(A)-containing RNA than the sucrose gradient approach. The most striking case is shown by the  $\alpha$ -FP standard curve which ranges from 1 pg to 1 ng. In this case a total of  $4 \times 10^{-13}$  g of  $[^3\text{H}]$ poly(dT) ( $\sim 40$  cpm) was reacted in 0.01 mL of 1 M NaCl with  $1 \times 10^{-12}$  g of  $\alpha$ -FP mRNA which contains  $\sim 5 \times 10^{-14}$  g of poly(A). Because the concentration of  $[^3\text{H}]$ poly(dT) was so low, the time of incubation at room temperature was increased to 7 h. About 20% of the input counts per minute adsorbed to the HA in these experiments. This is equivalent to  $\sim 7-8$  cpm. The  $2\sigma$  background of the counting system used was  $16 \pm 0.9$  cpm (four different background vials, each counted for 45 min and the data averaged). The data point presented in Figure 5 for 1 pg of  $\alpha$ -FP RNA is an average of four different determinations. Each of the four separate determinations for the 1-pg point was counted for 65 min. The individual counts per minute values ranged from 21.9 cpm (1423 total counts) to 24.5 cpm (1593 total counts). After the background ( $16 \pm 0.9$  cpm) was subtracted, these samples had  $2\sigma$  standard deviations counts per minute values of  $5.9 \pm 1.2$  and  $8.5 \pm 1.2$  cpm. The average  $2\sigma$  standard deviation value was  $7 \pm 1.2$  cpm for the four determinations. A similar analysis was done for the 0.01-ng values for the polio RNA. The data point presented in Figure 5 for 0.01 ng of polio RNA is an average of six separate determinations. The range of values obtained for average points is shown by the solid lines above and below each point.

Table IV: Quantitation of Poly(A) by HA and S-1 Nuclease<sup>a</sup>

RNA	assay method	large	small
		[ <sup>3</sup> H]poly(dT) [ng of [ <sup>3</sup> H]-poly(dT) per ng of RNA in hybrid]	[ <sup>3</sup> H]poly(dT) [ng of [ <sup>3</sup> H]-poly(dT) per ng of RNA in hybrid]
long poly(A) (greater than 280 nucleotides)	S-1	0.88	0.93
	S-1	1.0	
	HA	2.1	1.6
	HA	1.8	
28 nucleotide long poly(A)	HA	1.9	
	S-1	0.99	0.84
	S-1	0.98	
	HA	2.6	1.6
polio type 2	HA	2.4	
	HA	2.4	
	S-1	0.006	
	S-1	0.006	
	HA	0.012	0.0085
	HA	0.013	0.011
	HA	0.013	

<sup>a</sup> The HA and S-1 nuclease methods are described under Materials and Methods. Large [<sup>3</sup>H]poly(dT) (preparation no. 3 in Table I) reacted with an excess of poly(A) and assayed by HA and S-1 nuclease gave respectively 98 and 93.7% hybridization. Large [<sup>3</sup>H]poly(dT) reacted by itself and assayed by HA and S-1 nuclease gave respectively 1.3 and 2.4% hybridization. Small [<sup>3</sup>H]poly(dT) was the result of preparation no. 2 (Table I) aging. Small [<sup>3</sup>H]poly(dT) reacted with an excess of poly(A) and assayed by HA and S-1 nuclease gave 40 and 26% hybridization, respectively. The self-reaction of the small [<sup>3</sup>H]poly(dT) was 3.6 and 5.0% as measured by HA and S-1 nuclease, respectively. All reactions with large [<sup>3</sup>H]poly(dT) were done at [<sup>3</sup>H]poly(dT)/poly(A) ratios of 4-7:1. Reactions with the small [<sup>3</sup>H]poly(dT) were done at [<sup>3</sup>H]poly(dT)/poly(A) ratios of 12-15:1. The large and small [<sup>3</sup>H]poly(dT) molecules were respectively 150-200 and 30-40 nucleotides as measured by alkaline sucrose sedimentation.

Figure 5 also presents a polio RNA calibration curve done by assaying with S-1 nuclease in a manner similar to that described by Fan & Baltimore (1973). There exists an essentially linear relationship between the amount of RNA and the quantity of [<sup>3</sup>H]poly(dT) hybridized to the RNA. The slope of the S-1 nuclease line in Figure 5 is 1.03. The sensitivity of poly(A) detection of this method should be similar to that of the HA method.

**Quantitation of Poly(A) Present in RNA.** The poly(A) present in an RNA preparation can be quantitated by saturating the poly(A) with [<sup>3</sup>H]poly(dT) and then determining the amount of poly(dT) hybridized to the RNA. The most accurate quantitation values are obtained by the S-1 nuclease method. Table IV compares the S-1 nuclease and HA methods. Known amounts of short (28 nucleotides long on average) and long (greater than 300 nucleotides long) poly(A) were reacted with an excess of large (150-200 nucleotides long) or small (30-40 nucleotides long) [<sup>3</sup>H]poly(dT). These mixtures were then assayed by S-1 nuclease and HA. The mass of [<sup>3</sup>H]poly(dT) hybridized was calculated from the specific radioactivity of the [<sup>3</sup>H]poly(dT), and the mass ratio of [<sup>3</sup>H]poly(dT) to poly(A) in the poly(A)-poly(dT) hybrid was determined. The ratios obtained with the S-1 nuclease method were close to the ideal value of 1. These ratios ranged from 0.84 to 1. In contrast, the ratios obtained with the HA method ranged from 1.6 to 2.6. This high ratio indicates that extensive regions of single-strand poly(dT) are present in the poly(A)-poly(dT) hybrid. These regions are destroyed in the S-1 nuclease assay.

It is clear that the S-1 nuclease method is the preferred method for quantitating poly(A). Table IV also presents the

[<sup>3</sup>H]poly(dT)/RNA ratios for poly(A)-poly(dT) hybrids formed when [<sup>3</sup>H]poly(dT) was reacted with polio type 1 RNA. The S-1 nuclease data indicate that 0.6% of the polio RNA is poly(A). The HA method yields about twice this value. If it is assumed that all of the poly(A) present in this RNA preparation is attached to the polio RNA, it can be calculated that each polio RNA contains on the average 45-50 A residues. This value is comparable to those obtained by other workers for picornavirus RNA (Yogo & Wimmer, 1972; Hruby & Roberts, 1976).

### Discussion

The specific radioactivity of the [<sup>3</sup>H]poly(dT) limits the sensitivity of detection of free poly(A) and poly(A)-containing RNA. The [<sup>3</sup>H]poly(dT) used here had a specific radioactivity of  $3.4 \times 10^8$  dpm/ $\mu$ g. At a 36% counting efficiency, this corresponds to  $\sim 1 \times 10^8$  cpm/ $\mu$ g. Higher specific radioactivity poly(dT) can be produced by synthesizing the poly(dT) with [<sup>32</sup>P]dTTP or by <sup>32</sup>P end labeling poly(dT) with polynucleotide kinase (Richardson, 1971). Radioactive poly(dT) of a defined average size can also be produced by including an appropriate quantity of dideoxythymidine triphosphate in the poly(dT) synthesis mixture.

The [<sup>3</sup>H]poly(dT) is useable for a period of 6-12 months; the chain length becomes shorter with time. Even [<sup>3</sup>H]poly(dT) 30-40 nucleotides long on average can be used to detect and quantitate poly(A), although when tested only  $\sim 40\%$  of it hybridizes to an excess of poly(A). This small [<sup>3</sup>H]poly(dT) has an advantage in that it does not promote multimer formation when reacted with an excess of polio RNA (data not shown). If quantitating RNA with such small [<sup>3</sup>H]poly(dT), it is important to use at least a sixfold excess of the reactable poly(dT). A 6-fold excess of reactable small poly(dT) is equivalent to a 15-fold excess of the total population of small poly(dT).

The hybridization of [<sup>3</sup>H]poly(dT) to poly(A) occurs very rapidly at room temperature in 0.5 M NaCl even at very low concentrations. The RNA attached to the poly(A) tract does not appear to greatly affect the rate of reaction of poly(dT) with the poly(A). Similar rates of hybridization are seen for the reaction of an excess of poly(dT) with long ( $C_{0t1/2} = 1 \times 10^{-6}$  mol s L<sup>-1</sup>) and short ( $C_{0t1/2} = 8 \times 10^{-7}$  mol s L<sup>-1</sup>) poly(A) (Table I). The kinetics are approximately pseudo first order in form. The poly(A) attached to polio RNA reacts at about the same rate ( $C_{0t1/2} = 1 \times 10^{-6}$  mol s L<sup>-1</sup>; this assumes a polio RNA content of 0.6%).

The hybridization of radioactive homopolymers has been used by others (Rosbash & Ford, 1974; Sawin et al., 1977) to size poly(A)-containing RNA by first sedimenting the RNA into a sucrose gradient, then fractionating the gradient, and then hybridizing each fraction to the homopolymer. The sucrose method described here is much easier since it involves only one hybridization reaction. Since the single-strand [<sup>3</sup>H]poly(dT) is small, nonhybridized [<sup>3</sup>H]poly(dT) does not migrate far into the gradient while the hybridized [<sup>3</sup>H]poly(dT) sediments with the RNA to which it is attached.

Very small quantities of RNA can be sized and quantitated by using this procedure. A 0.3-ng amount of sedimented polio RNA resulted in a clearly visible peak containing  $\sim 500-600$  cpm. Polio contains  $\sim 0.6-1\%$  of its RNA as poly(A). With this same design, a peak containing 100-200 cpm is readily detectable. This would be equivalent to detecting  $\sim 0.05-0.1$  ng of polio RNA. A key to detecting even smaller amounts by this method is the ability to separate the RNA peak from the unreacted [<sup>3</sup>H]poly(dT) in order to have no overlap between these two classes. This can be done in several ways.

One may use a longer tube for the gradient analysis, or the hybridized [ $^3\text{H}$ ]poly(dT)-RNA may be separated from the nonhybridized [ $^3\text{H}$ ]poly(dT) before the gradient analysis with HA. Higher specific radioactivity poly(dT) may also be used. A 10-fold increase would result in a 10-fold greater detection sensitivity.

The sucrose gradient, HA, and S-1 nuclease methods can all be used to quantitate poly(A)-containing RNA. Standard curves constructed using purified RNAs are shown in Figures 4 and 5. The slopes of these curves demonstrate that, over a 100-1000-fold range in RNA concentration, the quantity of [ $^3\text{H}$ ]poly(dT) hybridizing to an RNA increases proportionally with the amount of RNA. The linear and proportional character of these curves show that the [ $^3\text{H}$ ]poly(dT)-poly(A) hybridization reaction is behaving according to theory.

A standardization curve should be constructed for each preparation of [ $^3\text{H}$ ]poly(dT) and stock of purified RNA. When sucrose gradient or HA assays are used, the amount of [ $^3\text{H}$ ]poly(dT) hybridized to a particular RNA can vary, depending on the size of the [ $^3\text{H}$ ]poly(dT) and the integrity of the RNA. Table IV presents data regarding the effect of [ $^3\text{H}$ ]poly(dT) molecular weight on the amount of poly(dT) hybridized to polio RNA. Large [ $^3\text{H}$ ]poly(dT) (~150-200 nucleotides long) and short [ $^3\text{H}$ ]poly(dT) (~30-40 nucleotides long) were hybridized to large poly(A) (greater than 300 nucleotides long) and small poly(A) (average of 28 nucleotides long). Since some poly(A)-containing RNAs have longer poly(A) tracts than others, this study was designed to find out whether the size of the poly(dT) or the poly(A) would greatly influence the values obtained in poly(A) quantitation. The HA data in Table IV show that the [ $^3\text{H}$ ]poly(dT)-poly(A) hybrids contain 1.6-2.6 times more [ $^3\text{H}$ ]poly(dT) than poly(A). The ratio is higher for the large [ $^3\text{H}$ ]poly(dT)-poly(A) combinations and lowest for the small [ $^3\text{H}$ ]poly(dT) combinations. Therefore, small [ $^3\text{H}$ ]poly(dT) will give fewer counts per minute per nanogram of RNA than will larger poly(dT) when measured by HA or by sucrose gradient analysis.

When the S-1 nuclease assay is used, the poly(dT)-poly(A) ratio is not greatly affected by the poly(A) size or the [ $^3\text{H}$ ]poly(dT) size. The nuclease will remove any single-strand [ $^3\text{H}$ ]poly(dT) in the hybrid and may well destroy one strand of a two poly(dT)-one poly(A) triple-strand hybrid; RNase destroys one poly(U) strand of a two poly(U)-one poly(A) hybrid (Bishop et al., 1974). The poly(dT)-poly(A) ratios obtained with the S-1 nuclease method are close to 1 and range from 0.84 to 1.0. This indicates that S-1 nuclease is the method of choice for precise quantitation of poly(A).

The S-1 nuclease and HA methods provide a measure of the total amount of poly(A) present in an RNA preparation while the sucrose gradient method measures the poly(A) present in a particular size class of RNA. The sucrose gradient and HA methods should therefore give comparable counts per minute per nanogram of RNA values if all of the poly(A) present in an RNA preparation is attached to RNA of the proper size class. A lower sucrose gradient value may indicate that breakdown of the RNA has occurred. This situation should also be reflected in the sedimentation profile of the [ $^3\text{H}$ ]poly(dT)-labeled RNA. A lower value could also indicate the presence of free poly(A) or single-strand DNA. Higher organism DNAs contain ~0.5% poly(dT)-poly(dA) sequences (Shenkin & Burdon, 1972; Bishop et al., 1974). Care should be taken to remove DNA before assaying for poly(A).

The techniques presented here should prove useful for situations in which it is desirable to characterize much less than microgram quantities of polyadenylated RNA. We have

routinely used the sucrose gradient method to size nanogram amounts of polyadenylated RNAs prior to reverse transcription and in vitro translation, as well as specific polyadenylated RNAs annealed to [ $^3\text{H}$ ]poly(dT) as sedimentation markers in sucrose gradients. This labeling method has also been used to size  $\alpha$ -FP mRNA on neutral polyacrylamide gels and should be applicable to a wide range of gel techniques. In addition to characterizing purified RNA, we have developed a method for reacting [ $^3\text{H}$ ]poly(dT) with detergent-disrupted retroviral particles in order to quantitate and detect the presence of retrovirus RNA and viral cores. This method can detect the presence of low levels of virus in tissue culture media. The HA method is routinely used to obtain a rapid estimate of poly(adenylate) content of purified RNA. Other radioactive homopolymers can be used in a similar manner and will be useful in specific situations. Gillespie et al. (1972) reported the use of [ $^3\text{H}$ ]poly(C) to detect poly(guanylate) tracts present in polio virus RNA. We have used [ $^3\text{H}$ ]poly(G) to size EMC RNA (data not shown) which possesses an internal poly(cytidylate) tract.

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## Multistep Mechanism of Codon Recognition by Transfer Ribonucleic Acid<sup>†</sup>

Damian Labuda<sup>\*†</sup> and Dietmar Pörschke\*

**ABSTRACT:** The mechanism of codon recognition by tRNA is investigated in the system tRNA<sup>Phe</sup> + UUC by temperature-jump measurements using the Wye base fluorescence as a label. In 0.4 M Na<sup>+</sup> and 5 mM Mg<sup>2+</sup> a two-step reaction is observed and described quantitatively; UUC is shown to bind preferentially to one of two conformations on the anticodon loop. In 0.1 M Na<sup>+</sup> and 10 mM Mg<sup>2+</sup> an additional relaxation

effect is observed, which indicates a codon-induced conformation change leading to an association of tRNA molecules. The codon-induced tRNA association is demonstrated independently by equilibrium sedimentation. The present results suggest a more active role of tRNA during translation than anticipated.

The main step in the translation of a genetic message is the recognition of codons by adaptor molecules, the tRNAs. Compared to the codons, the adaptors are large and complex structures consisting of ~80 nucleotide residues and yet using only 3 of them for *direct* interaction with the codon. There are several reasons for the rather high complexity of tRNA molecules. tRNAs have to provide recognition sites during numerous biological activities in which they participate (Clark, 1977). The following question arises, however. (1) Is tRNA a passive structure, which exposes a variety of sites for interaction with different components of the cell, or (2) does tRNA participate more actively in the decoding process? In the second case the whole tRNA structure could modulate the interaction between codon and anticodon triplets and, conversely, the interaction between codon and anticodon could trigger some response in the remote parts of the molecule.

In recent years much conflicting evidence has been presented concerning different conformations of tRNAs and their function during translation. Two structural transitions are mainly under discussion.

The anticodon loop of tRNA may convert from the 3'-stacked conformation, found in crystals [for review, cf. Rich & RajBhandary (1976) and Clark (1977)], to the 5'-stacked conformation. This transition, proposed by Fuller & Hodgson (1967), may be important during protein biosynthesis (Woese, 1970). Recently, a relaxation process observed in tRNA<sup>Phe</sup> has been assigned to the conversion between the 3'- and 5'-stacked conformations (Urbanke & Maass, 1978).

The second transition is the unfolding of the TΨC loop induced by the codon-anticodon interaction (Ofengang & Henes, 1969; Shimizu et al., 1969). The TΨC loop is usually hidden due to tertiary interactions (Rich & RajBhandary,

1976). According to oligonucleotide binding data, the TΨCG sequence becomes exposed upon complex formation between codon and anticodon (Möller et al., 1979). Evidence for a codon-induced rearrangement of tRNA structure has also been obtained from chemical modification experiments (Wagner & Garrett, 1979). Other measurements using different techniques did not give any evidence for codon-induced unfolding of tRNA (Yoon et al., 1975; Geerdes et al., 1978; Grosjean et al., 1976; Davanloo et al., 1979; Geerdes, 1979).

In the present investigation the binding of UUC to its cognate tRNA<sup>Phe</sup> is studied by temperature-jump spectroscopy and equilibrium centrifugation. The fluorescence of the Wye base, a native label located adjacent to the 3' side of the anticodon triplet (RajBhandary & Chang, 1968), is used to follow the reaction. The results indicate a four-step mechanism of codon recognition, involving two conformational changes and dimerization of tRNA. It shows that tRNA is not a passive adaptor but actively participates in the process of codon reading.

### Materials and Methods

tRNA<sup>Phe</sup> from yeast and ApApA were purchased from Boehringer, Mannheim. The tRNA specific acceptor activity was 1.1 nmol/A<sub>260</sub> unit. Highly pure tRNA<sup>Phe</sup> samples (specific activity of 1.5 nmol/A<sub>260</sub> unit) were kindly given by Drs. M. Sprinzl and H. Faulhammer from Max-Planck-Institut für experimentelle Medizin, Göttingen. The codon oligonucleotide UpUpC was synthesized as described by Sprinzl et al. (1976). tRNA<sup>Phe</sup> samples were dissolved in buffer AB (10 mM Tris-cacodylate, 400 mM NaClO<sub>4</sub>, and 5 mM Mg(ClO<sub>4</sub>)<sub>2</sub>, pH 7.1) or in buffer ABT (50 mM Tris-cacodylate, 100 mM NaClO<sub>4</sub>, and 10 mM Mg(ClO<sub>4</sub>)<sub>2</sub>, pH 7.2) and annealed at 65 °C for 5 min as described by Grosjean et al. (1976). For measurements of Mg<sup>2+</sup> concentration (c<sub>Mg</sub>) dependence, the c<sub>Mg</sub> was varied in ABT buffer as indicated. For c<sub>Mg</sub> = 0, 0.5 mM Na<sub>3</sub>EDTA was added instead of Mg<sup>2+</sup>-salt. The tRNA<sup>Phe</sup> concentration (c<sub>tRNA</sub>) was calculated on the basis of its acceptor activity. The concentrations of UpUpC (c<sub>UUC</sub>)

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# Biochemistry



## Extent of Double Strandedness in Avian Myeloblastosis Virus RNA

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The extent of double strandedness of avian myeloblastosis virus 70S RNA has been determined from fluorescence measurements of the intercalation of ethidium bromide. We have shown that 50% of the nucleotides of 70S RNA in solution are in a stable helical configuration. This value does not include small helical regions that are too unstable to permit intercalation of the dye. The avian myeloblastosis virus RNA as it exists within the virion has the same degree of helicity as the free 70S RNA. Heating the free 70S RNA to 55 or 70 °C, followed by cooling, does not measurably change the degree of helicity; the subunits therefore have as much helicity as the parent molecule.

The RNA of oncornaviruses consists primarily of a high-molecular-weight species sedimenting in the 60 to 70S range (5, 6, 16). It is apparently composed of three or four large subunits which dissociate upon heating or on treatment with dimethylsulfoxide (1, 5, 15) or formamide (19). It is generally assumed that the subunits are held together by hydrogen bonds, giving rise to double-helical regions in the molecule. However, the extent of the helicity and the conformation of the 70S RNA are uncertain, both within the virion and after isolation.

Estimates of the helical content of the RNA have been made from its resistance to the specific single-strand nucleases (8, 9, 13, 14, 20). The main disadvantage of this procedure is that these enzymes have not been fully characterized. It is not known, for example, how long a helical region is required for resistance or what are the effects of internally melted or mismatched regions. To determine the helical content by a more precise procedure, we have used fluorescence spectroscopy. Ethidium bromide (EB) fluoresces intensely when it is intercalated between the base pairs of either RNA or DNA helices, but not when it binds to single-stranded regions (2, 11). We have been able to determine on this basis the helical content of isolated 70S RNA directly from fluorescence measurements. With the same methodology, we have also determined the helical content of the RNA as it exists within the virion. We have also shown that heating and then cooling of isolated 70S RNA does not lead to a decrease in helicity, implying that nearly all the helicity is within rather than between the subunits.

### MATERIALS AND METHODS

**Materials.** Avian myeloblastosis virus (AMV) was supplied as plasma from infected chickens by Life Sciences, Inc. (St. Petersburg, Fla.).

Purified Reo virus double-stranded RNA (over 99% double-stranded) was kindly donated by M. Kinsham (Roche Institute of Molecular Biology, Nutley, N.J.).

28S rRNA isolated from rat liver was a generous gift of M. G. Hamilton. 4S RNA was purchased from General Biochemicals Div. (Mogul Corp., Chagrin Falls, Ohio). EB (grade B) was purchased from Calbiochem (Los Angeles, Calif.).

**Virus and RNA isolation.** Plasma was stored at -70 °C and was thawed just prior to use. All operations were performed at 0 to 4 °C. After thawing, the plasma was filtered through four layers of gauze and layered on top of a glycerol cushion, and virus was pelleted onto the cushion by centrifugation at 25,000 rpm for 1 h in an SW27 rotor. The virus was collected from the cushion, diluted threefold with buffer containing 0.01 M Tris-hydrochloride (pH 7.8), 0.15 M NaCl, and 0.01 M EDTA (TNE buffer), and layered over linear 20 to 50% (wt/vol) sucrose gradients prepared in TNE buffer. AMV was isopycnicly banded at 105,000 × g in an SW27 rotor for 15 h at 4 °C, at which time the virus band was collected, suspended in TNE buffer, and pelleted at 150,000 × g at 4 °C for 30 min. 70S RNA was isolated by the method of Kacian et al. (12).

**Methods.** Fluorescence measurements were made with a Cary 50-026-900 differential recording spectrofluorimeter with front surface illumination at an angle of 23° between the exciting beam (546 nm) and the emitted light (602 nm). The illuminating light is broken up by a chopper and passed alternately through a rhodamine quantum detector (located in the sample compartment) and the sample. The signal displayed on the recorder is the ratio between these two signals, thus correcting for variation of light

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intensity of the source. The ratio ( $V$ ) of fluorescence intensity of bound EB to the intensity of free dye was determined for each RNA at 546 nm. For 28S rRNA and 4S RNA it was 50; for Reo and AMV RNA it was 70. No corrections for light scattering were found necessary at the concentrations and instrumental settings used in these experiments. Measurements were made on 1.1 ml of solution in a cuvette (2-mm path length). EB at a concentration of about  $10^{-7}$  M was added to the cuvette by an automatic pipette capable of delivering 1 to 200  $\mu$ liters. The concentration of RNA in terms of phosphate was in the range of  $1 \times 10^{-6}$  to  $10 \times 10^{-6}$  M. The buffer was a mixture of 0.1 M NaCl and 0.05 M Tris (pH 7.8). Measurements were made at 25 C. The combined instrumental and experimental error was generally less than 2%.

## RESULTS

The number ( $n$ ) of EB-binding sites per nucleotide phosphate, and the strength of binding ( $k$ ) are given by  $r/c = kn - kr$ , where  $r$  is the number of EB molecules bound per nucleotide phosphate at a free EB concentration of  $c$ . Clearly,  $n$  is the limiting value of  $r$  at infinite EB concentration. EB shows significant fluorescence only when it intercalates between base pairs. Theoretically, a maximum of one EB molecule can be bound per two phosphate groups (i.e.,  $n = 0.5$ ), assuming that intercalation can occur between each pair of base pairs. Because of steric reasons, the actual number bound will always be substantially less than this.

The value of  $r$  is calculated from the intensity of fluorescence,  $I_b$ , in the presence of RNA, as follows:  $r = I_b/(k \cdot P)$ , where  $k = I_b/V/c$ .  $I_b$  is the intensity of fluorescence in the absence of RNA, the other conditions being identical to those under which  $I_b$  is measured.  $C_0$  is the EB concentration (moles per liter),  $V$  is the ratio of intensities of bound and free EB, and  $P$  is the nucleotide phosphate concentration. (In a formal way,  $k$  is identical to an absorbance extinction coefficient.)

**Reo virus and 28S rRNAs.** Reo virus and 28S rRNAs have been chosen as models of known structure to provide a basis for estimating the degree of helicity of 70S RNA. For this reason, we have determined complete EB-binding curves for both RNAs (Fig. 1). The value of  $n$  (the number of EB molecules bound per phosphate at saturation) for Reo virus RNA is 0.044, indicating that one EB molecule is intercalated in the 100% double-stranded Reo virus RNA per 11.4 base pairs, or one EB per 23 phosphate groups. The value of  $n$  for 28S rRNA is 0.012, indicating that 28S rRNA intercalates 26% as much EB as the completely helical Reo RNA. The data for both nucleic acids are

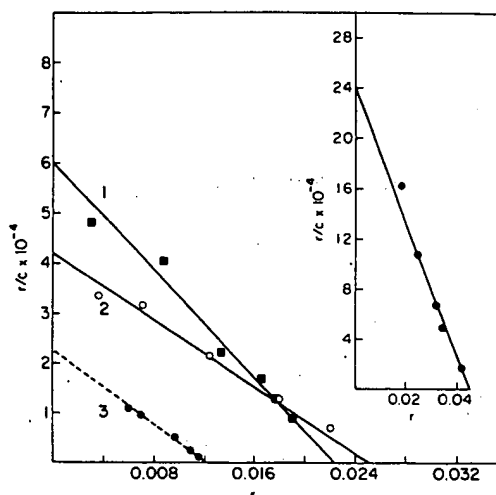


FIG. 1. Binding of EB to various RNAs. Curve 1: Isolated 70S RNA at a concentration of  $1.32 \times 10^{-6}$  M phosphate. The concentration of EB ranged from  $10.7 \times 10^{-7}$  M to  $214 \times 10^{-7}$  M tris-0.1 M NaCl (pH 7.8). Curve 2: AMV-disrupted virions in buffer containing 0.1% NP-40. The phosphate concentration was  $1.4 \times 10^{-6}$  M. The EB concentration was the same as that for curve 1. Curve 3: 28S rRNA at a concentration of  $2 \times 10^{-6}$  M; EB concentration range  $10.7 \times 10^{-7}$  to  $160 \times 10^{-7}$  M. Inset: Reo virus RNA at a concentration of  $4.1 \times 10^{-6}$  M; EB concentration range  $8.5 \times 10^{-7}$  to  $64 \times 10^{-7}$  M.

represented by a linear plot showing unique values for  $k$  (a measure of the strength of binding) and  $n$  in each case. Table 1 summarizes the  $k$  values and the number of phosphate groups per EB molecule (number is equal to  $1/n$ ) for all RNAs examined.

**AMV RNA.** The results for the binding of EB to isolated 70S AMV RNA are shown in Fig. 1 (curve 1). The data are fit best with a straight line, which indicates that there is only one set of binding sites with a single binding constant. This means that all intercalation sites are essentially indistinguishable. The value for  $n$  is 0.022, or 1 EB per 45 phosphate groups. The same binding curve was obtained whether the RNA was used directly from the sucrose gradient or first precipitated with alcohol and redissolved.

To test the possibility that structural changes might occur or that factors might be lost during the isolation of the 70S RNA, the same experiment was carried out with the intact virus and with Nonidet P-40-disrupted virions. The NP-40 dissociates most of the protein from the RNA, leaving a "core" structure containing some protein. The results should show whether the mere act of disruption gives rise to impor-

TABLE 1. Summary of experimental values for the RNAs studied

Sample	<i>n</i>	No. of phosphates <sup>a</sup> /EB molecule	<i>k</i> × 10 <sup>-4</sup> <sup>b</sup>
Isolated 70S RNA	0.022	45	2.7
Disrupted virion 70S RNA	0.025	40	1.7
70S RNA in virion <sup>c</sup>		44	ND <sup>d</sup>
Isolated 70S RNA (pre-heated to 55 C)	0.022	45	2.1
Isolated 70S RNA (pre-heated to 70 C)	0.022	45	2.1
28S rRNA	0.012	84	1.9
Reo virus RNA	0.044	22	5.5

<sup>a</sup> The number of phosphate groups per EB molecule is the reciprocal of *n*, the value of *r* at the abscissa in Fig. 1 and 2.

<sup>b</sup> *k* is obtained by dividing the ordinate intercept by *n*.

<sup>c</sup> 10  $\mu$ liters of a suspension of AMV (1.6 mg of protein per ml, 0.05 M Tris-0.1 M NaCl [pH 7.8]) was added to 1.1 ml of  $2.47 \times 10^{-7}$  M EB in the same buffer. The final 70S RNA phosphate concentration was  $1.2 \times 10^{-6}$  M.

<sup>d</sup> ND, Not determined.

tant structural changes affecting the intercalation of EB. However, before doing this we had to determine the contribution to the intensity of fluorescence of the other RNAs which are present in the virion in addition to the 70S RNA: the 4, 5, 7, 18, and 28S RNA components. Most of the non-70S RNA is of the 4S type; we found that one EB intercalates per 75 phosphate groups in 4S RNA (data not shown). This is in agreement with the results of Tao et al. (18). Thus, we were able to correct for the small increment in the intensity of fluorescence due to this irrelevant RNA (which amounts to 20% of the total RNA in the virus [7]). The extent of intercalation in 28S rRNA is also low, as discussed above. We did not determine the binding of EB to the 5, 7, or 18S RNAs, but since these RNAs comprise only about 8% of the total RNA, their contribution to the total fluorescence is low and can be neglected. We estimate this contribution to be about 2%, assuming that the extent of binding is about the same as for the 4S and 28S RNAs.

The results for the binding of EB to 70S RNA in NP-40-disrupted virions (Fig. 1, curve 2) again are fit best by a straight line, indicating that there is only one set of binding sites. The value for *n* (0.025), however, is slightly higher than that for isolated 70S RNA. We do not know

the significance of this difference, if any.

In the determination of a complete binding curve, increasing amounts of EB are added to a fixed concentration of RNA and then are extrapolated in both directions to infinite EB concentration and zero EB concentration to obtain *n*, the number of binding sites, and *k*, the binding constant. In the case of the undisrupted virus, the RNA content of the virion is low, so that the amount of RNA required for binding studies at low EB concentrations produces a turbidity too high to permit meaningful measurements. That is, nearly all of the fluorescence is lost by light scattering. It is thus impossible to determine *k*. However, the value of *n* can be determined with a small quantity of virus and a large excess of EB. This procedure is tantamount to a direct determination of the number of EB molecules bound at infinite EB concentration. The value of *n* determined for 70S RNA within the virion is 0.022, identical to that for isolated 70S RNA (Table 1). This indicates that the secondary structure of the 70S RNA does not change upon isolation.

**Heated 70S RNA.** Heating of 70S RNA results in two specific changes (3). At 55 C at this ionic strength the molecule dissociates into 35S subunits, which are still active as primer-templates in assays in vitro. At 70 C, virtually all of the 70S dissociates into 35S subunits and apparently small (<10S) primer RNAs are dissociated from these subunits with a concomitant loss of template activity. 70S RNA samples were heated to each of these temperatures in the cuvette and were cooled quickly in ice to minimize reannealing. The binding of EB to the heat-treated RNAs is shown in Fig. 2, where it is evident that the curves for the samples heated either to 55 C or 70 C are identical to that for unheated 70S RNA. Any destruction of helicity at 55 or 70 C is therefore largely reversible.

## DISCUSSION

We have determined the binding constants and the number of sites at which intercalation of EB occurs for AMV 70S RNA in various physical states, using fluorescence measurements to indicate intercalation. It has been established that fluorescence results from intercalation of the EB molecule between base pairs present in a helical structure, and not from binding to single-stranded nucleic acids (11). Our results are summarized in Table 1. The calculation of these parameters does not depend on any assumptions. The fact that all the data are expressible by straight lines shows that there is only one type of binding site, associated

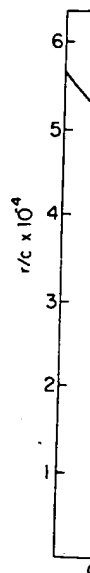


FIG. 2. RNA. Sy<sup>+</sup> heated to as in Fig. heated at then quick 25 C.

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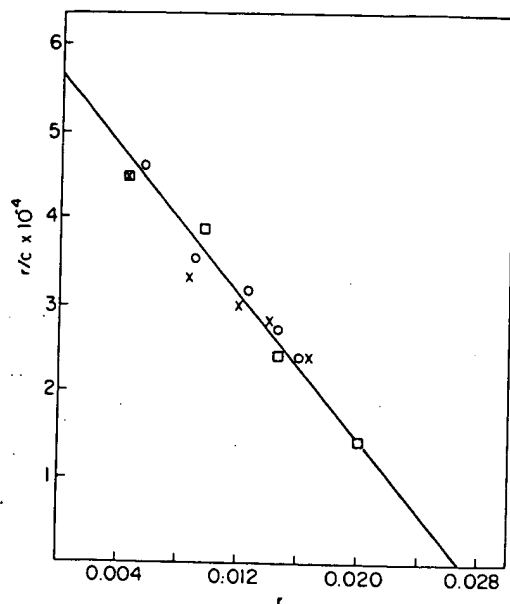


FIG. 2. Binding of EB to heated and unheated 70S RNA. Symbols: ○, unheated; □, heated to 55°C; ×, heated to 70°C. The RNA and EB concentrations were as in Fig. 1. The cuvette containing the solution was heated at the indicated temperature for 3 min and then quick-cooled in ice. Measurements were made at 25°C.

with the fluorescence phenomenon. This has been reported earlier both for DNA and RNA (11).

By comparing the number of binding sites for 70S RNA to that for Reo virus RNA, which is completely double-stranded, we calculate the degree of helicity of the AMV 70S RNA to be 50%. Because the molecular weights of these RNA molecules are high, differences in molecular weight do not affect the binding properties (11). However, this value may be a minimum if there are also double-stranded regions in the 70S molecule which are too short to bind EB. We have assessed this situation by comparing the degree of helicity of 28S rRNA calculated by different methods. The fluorescence measurements reported here indicate that 26% of 28S RNA is double stranded by comparison with Reo virus RNA. However, it has been shown by various other physical methods that about 60% of this RNA is helical (17), with regions that have been estimated to be 4 to 17 base pairs long (4). We have shown (L. F. Cavalieri, unpublished data) that helical regions equal to or larger than 10 base pairs bind EB. We deduce then that helical regions which are about 4 to 7 base pairs long do not bind EB or do so very

weakly, since 58% of the helicity of 28S rRNA is not observed by fluorescence. Any similar short helical regions in 70S RNA would not be registered in the fluorescence measurements. The 50% helicity observed must therefore correspond to longer stretches which are not transient, thus implying a degree of complementarity which is significant with regard to the information content of the RNA. Nearly all the helicity must be internal rather than between subunits, since it is reversible, as shown by the heating results. We are investigating this structural aspect of the subunits, since it is undoubtedly important in the role of the subunits as RNA messengers, as well as in their reverse transcription.

#### ACKNOWLEDGMENTS

I thank Elizabeth Carroll for expert technical assistance, Barbara Hatch Rosenberg for helpful discussions, and Stuart Marcus and Mukund Modak for isolation of the AMV RNA.

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*It is characteristic of  
Science and Progress  
that they continually  
open new fields to  
discussion.* — K. S. R. R.



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## Rapid Diagnosis of Rotavirus Infection by Direct Detection of Viral Nucleic Acid in Silver-Stained Polyacrylamide Gels

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A rapid simple technique for the diagnosis of rotavirus has been developed based on the sensitive detection of rotavirus double-stranded RNA genome segments separated in polyacrylamide gels. The method utilizes a recently described ultrasensitive silver stain for polypeptides, which can also detect subnanogram amounts of nucleic acid. The sensitivity of the technique is comparable with that of electron microscopy or enzyme-linked immunosorbent assay.

Rotaviruses cause enteritis in a wide variety of species and have been intensively investigated for more than a decade (reviewed 5, 14). As members of the *Reoviridae* (13), they have a double-stranded RNA (dsRNA) genome consisting of 11 segments ranging in molecular weight from approximately  $2.0 \times 10^6$  to  $0.2 \times 10^6$  (9). Electrophoretic analysis has revealed major differences in the mobility of the genome segments between virus isolates from different host species and minor differences between individual isolates from the same species. Thus, genome electrophoretotyping has been the most common method for both taxonomic and epidemiological studies (9, 12, 18).

Recently, several methods have been reported which utilize silver staining for the ultrasensitive detection of polypeptides resolved by polyacrylamide gel electrophoresis (PAGE) (16, 17, 20). We used one of these methods (20) to monitor the purification of rotavirus from feces and found that the dsRNA bands were also stained with high sensitivity. This result was consistent with the well-described property of silver ions to form a stable complex with nucleic acids (7). Similar nucleic acid staining with another silver staining method has recently been reported by Somerville and Wang (22).

In this communication we describe a diagnostic test for rotavirus in feces based on this ultrasensitive detection of viral dsRNA, which has the advantages of simplicity, economy, and speed, and which simultaneously identifies the electropherotype.

### MATERIALS AND METHODS

**Fecal specimens.** Infected and control fecal specimens were obtained from cattle and human sources. The bovine samples and two of the human samples

were examined for rotavirus by electron microscopy (EM) and by enzyme-linked immunosorbent assay (ELISA). The ELISA was performed essentially by the method of Yolken et al. (25), using a hyperimmune rabbit serum raised against tissue culture-grown bovine rotavirus, with a neutralization titer of 1:10,240 both to coat the wells and as a conjugate to detect antigen. Unconcentrated samples were examined by EM as described by Snodgrass et al. (21). The samples in the dilution experiment were coded and scored blind. The majority of the human specimens were kindly provided by the Edinburgh Regional Virus Laboratory. Rotavirus diagnosis had been carried out on these specimens by the cell culture method of Bryden et al. (2).

**Nucleic acid extraction.** Fecal samples were diluted 1:4 by weight with 0.1 M sodium acetate buffer (pH 5.0) containing 1% (wt/vol) sodium dodecyl sulfate; the normal sample size used was 0.25 g of feces, which provided enough extract for at least 10 separate analyses. An equal volume of a 3:2 (vol/vol) 'phenol'-chloroform mixture was added to the fecal suspension, and the sample was mixed for 1 min. ('Phenol' consisted of a mixture of 500 g of phenol, 70 g of *m*-cresol, and 200 g of water containing 0.5 g of 8-hydroxyquinoline.) The emulsified mixture was then centrifuged for 10 min at  $1,200 \times g$ , and the resulting clear upper aqueous layer was removed. A sample was then prepared for electrophoresis by the addition of 10  $\mu$ l of 25% (wt/vol) sucrose containing 0.2% bromophenol blue to 40  $\mu$ l of the aqueous layer.

Occasional samples failed to yield sufficient clear aqueous layer, but it was found that either further centrifugation for 3 min at  $16,000 \times g$  in a microcentrifuge (Mechanika Preczyjna, type 320a) or the addition of 0.5 ml of buffer followed by remixing and centrifugation at  $1200 \times g$  gave an ample clear layer.

**PAGE.** The 50- $\mu$ l samples were loaded onto 5% polyacrylamide slab gels (acrylamide-to-bis-acrylamide ratio of 37.5:1) which were polymerized with 0.01% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine and 0.05% (wt/vol) ammonium persulphate. The gel and electrode buffer was 0.036 M Tris-0.03 M

sodium dihydrogen phosphate-0.001 M EDTA (pH 7.8). Gel dimensions were 14-cm wide by 19-cm long and 0.15-cm thick. It should be noted that the gel thickness is critical with the silver staining technique (20). Deep sample wells (0.6 by 2.0 cm) facilitated loading without the transfer of sample to neighboring wells. Electrophoresis was performed at room temperature for 16 h at 20 mA and 70 V. In most experiments, one of the glass plates used to form the gel mold was treated with a 1% solution of Silane 174A in ethanol (Union Carbide Corp.) for 10 min, dried in air, rinsed in distilled water, and redried. This treatment caused the gel to adhere strongly to the plate and greatly simplified its handling during staining.

**Silver staining.** The gels were stained by using a slight modification of the method of Sammons et al. (20). The initial fixation steps described for protein staining were omitted, and the gels were washed with 10% ethanol-0.5% acetic acid for 30 min and then soaked in 0.011 M silver nitrate for 2 h. The gel was then rinsed briefly in distilled water, and the reduction step was performed with a solution of 0.75 M sodium hydroxide containing 0.1 M formaldehyde and 0.0023 M sodium borohydride. The bands appeared at this stage, and the reduction was continued until the bands were clearly visible for a maximum of 10 min. In our early experiments the gels were then placed in 0.07 M sodium carbonate, and the intensity of staining of both the bands and the background increased slightly in the 20 min or so after transfer. After 30 min, the gels were placed in fresh carbonate solution. However, it was found that, when using gels which were stuck to a glass plate with Silane 174A, an unacceptable degree of background staining sometimes developed when the gel was placed in the carbonate solution. This could be prevented by treating the gel with a 5% acetic acid solution for 30 min after the reduction and then transferring the gel to carbonate solution for storage. Gels have been successfully stored, sealed in polythene bags, for up to 6 months.

All of the solutions for the staining were made from single distilled water and, with the exception of the initial fixation solution, were degassed before use. The solutions were used in 200-ml volumes in a single plastic staining dish, and care was taken to avoid touching the gel surface with ungloved hands. Constant agitation of the solutions throughout the procedure was achieved with a rocking bed destainer. The gels were photographed by transmitted light, using a Wratten 85B filter.

**Purification of virion dsRNA.** Virus was purified from infected bovine feces essentially by the method described by Todd and McNulty (24), and the dsRNA was extracted with phenol and further purified by one cycle of CF11 cellulose chromatography (6) performed as described by Bevan et al. (1). The resulting dsRNA was quantified spectrophotometrically.

## RESULTS

The results obtained by direct extraction of feces with phenol followed by gel analysis of the extract are shown in Fig. 1. The first nine samples were all from a herd affected with enteritis, and eight may be clearly seen to contain the characteristic dsRNA segments of bo-

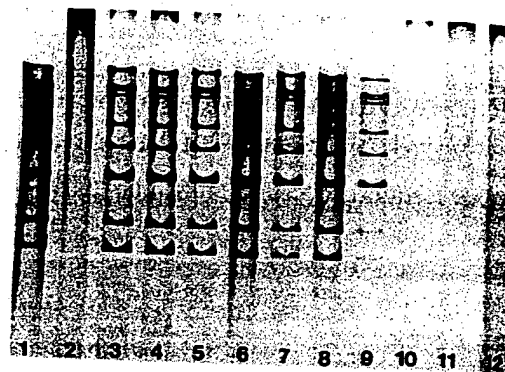


FIG. 1. Gel electrophoresis of fecal nucleic acid extracts. Tracks 1 through 9, extracts of fecal samples from diarrheic calves; tracks 10 through 12, extracts of feces from uninfected calves.

vine rotavirus; the other three control samples were negative. As would be expected, the samples from a single outbreak all showed the same electropherotype. Other bands were seen on the gels, especially near the origin where DNA forms a diffuse band, and occasional samples produced a continuous smear of stained material; but neither of these effects interfered with the detection of the dsRNA. The rotaviral genome segments could be identified by the characteristic sharpness and unique pattern of the dsRNA bands.

The results of a comparison between PAGE, ELISA, and EM are summarized in Table 1, together with the results of PAGE analysis on 24 human specimens which had been tested for rotavirus by cell culture. There was complete concordance between PAGE and ELISA results and only a single conflicting result in the PAGE and EM results. The level of virus in this one sample was clearly low, as the dsRNA bands were faint. The results with the human samples similarly showed just one conflicting result, which was positive by PAGE; this sample was obtained from a patient who also yielded other samples which were positive by cell culture.

The sensitivity of PAGE was investigated by dilution experiments. A positive sample judged to contain an average level of viral dsRNA was serially diluted with a negative sample to give a range of viral concentrations from 12.5 to 0.2% of that in the original sample, but with approximately the normal amount of contaminating non-viral material in each dilution. Extracts of these samples were analyzed by PAGE, and the results are shown in Fig. 2. Rotavirus dsRNA segments 1 through 4 were detected in dilutions down to 0.39%, but the lower-molecular-weight bands were not apparent at the higher dilutions. Figure 2 also shows the result of diluting the positive sample extract with electrophoresis

TABLE 1. Comparison of PAGE with other methods of rotavirus diagnosis

Specimen	No. of samples	Diagnostic method <sup>a</sup>				No. of samples in category
		PAGE	ELISA	EM	Cell culture	
Bovine	68	+	+	+	ND	41/68
		-	-	-	ND	26/68
		+	+	-	ND	1/68
Bovine	13	+	+	ND	ND	5/13
		-	-	ND	ND	8/13
Human	2	+	+	+	ND	1/2
		-	-	-	ND	1/2
Human	24	+	ND	ND	+	18/24
		-	ND	ND	-	5/24
		+	ND	ND	-	1/24

<sup>a</sup> +, Positive; -, negative; ND, test not performed.

buffer. All of the dsRNA segments were detected at a level of 0.39% of the original positive material.

A similar dilution series, using the same positive and negative feces and the appropriate buffers, was constructed and tested by ELISA and by EM (Table 2). EM was found to detect virus to a level of 1.56% of the original sample. The ELISA results are expressed as ratios of the positive and negative optical densities (P/N) as suggested by Yolken et al (25), who considered any value in excess of 2.1 to be rotavirus positive. Table 2 shows two values of P/N for each dilution. The first, and higher value, was based on the optical density given by the particular negative feces used for dilution, and the second is based on the optical density given by our standard uninfected feces. These data show the dilution series positive to the 0.39% and 1.56% levels, respectively. Thus, with the methods used, the sensitivities of PAGE, EM, and ELISA were approximately equal.

One further dilution series was investigated to determine the sensitivity of the silver staining method in absolute terms. Examination of gels loaded with a dilution series of purified dsRNA showed that the detection limit of the silver stain for a single band was 300 to 400 pg.

#### DISCUSSION

The diagnosis of rotavirus infection has been achieved by a variety of methods (reviewed in 10) based on either the direct visualization of the virion by EM or the detection of viral antigens by a wide diversity of immunological techniques, including the highly advanced and sensitive enzyme-linked fluorescence assay (26). The test described above is based on the direct extraction and detection of viral dsRNA. Two direct extraction methods have been reported previously, but both have been designed for epidemiological studies and genome analysis rather than for diagnosis. Clarke and McCrae (3)

described a method based on end labeling of total fecal nucleic acid followed by CF11 cellulose purification of the dsRNA and analysis by PAGE and autoradiography. This procedure could detect rotavirus dsRNA with very high sensitivity, but is too protracted and expensive to use as a routine diagnostic test. Theil et al. (23) recently reported a method for the bulk extraction of dsRNA from large fecal samples (6 ml), using CF11 cellulose as a batch procedure. Their results confirmed that directly extracted dsRNA was identical to virion dsRNA. It is an indication of the increased sensitivity of the silver staining method relative to ethidium bromide fluorescence that Thiel et al. (23) used the dsRNA from 0.4 ml of feces for a single analysis, whereas we routinely use the nucleic acid from only 0.01 ml of feces.

The greatest advantages of the PAGE and silver stain method are its lack of ambiguity and

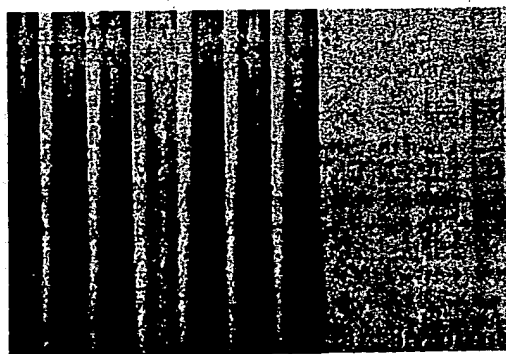


FIG. 2. Gel electrophoresis of fecal nucleic acid extracts. Tracks 1 through 7, extracts of a dilution series of bovine feces containing 12.5, 3.13, 1.56, 0.78, 0.39, 0.2, and 0% of positive sample, respectively; tracks 8 through 11, four dilutions in buffer of the nucleic acid extract of the positive feces containing 0.39, 0.78, 1.56, and 3.13% of the positive extract, respectively.

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TABLE 2. EM and ELISA results on the dilution series of positive feces<sup>a</sup>

Positive feces in dilution (%)	Test		
	EM	ELISA	
		P/N [1]	P/N [2]
12.5	+ [1]	9.12 (+)	4.8 (+)
6.25	+ [16]	5.55 (+)	2.92 (+)
3.13	+ [1]	4.79 (+)	2.52 (+)
1.56	+ [2]	4.25 (+)	2.23 (+)
0.78	—	3.53 (+)	1.85 (—)
0.39	—	2.28 (+)	1.20 (—)
0.20	—	1.86 (—)	0.98 (—)
0	—	1.0 (—)	0.53 (—)

<sup>a</sup> The figures in brackets indicate the number of viral particles found in a standard 10-min search. Positive-to-negative (P/N) ratios are explained in the text. The test results (+, —) are shown in parentheses.

the fact that it provides information about viral electropherotype. Since the test detects the viral genome which has a unique number and pattern of dsRNA segments the results are unequivocal. None of the samples we have examined to date has given any spurious bands which could be confused with viral dsRNA. The only problem encountered in our early trials of the technique was the accidental transfer of sample to a neighboring well in the gel at the time of loading; certainly care is required at this stage, and accurate loading is facilitated by the use of deep sample wells. If confirmation of a weak positive result is required, the sample may be concentrated very simply by ethanol precipitation.

The unambiguous nature of a positive PAGE test contrasts with the difficulties in the interpretation of low-positive values in the ELISA. False-positive results have been reported to occur with ELISA (27), and it has been necessary to incorporate pretreatment of the samples with mild reducing agents (27) or additional controls with blocking antiserum (25), as is our practice.

The fact that the PAGE and silver stain method simultaneously produces an electropherotype is a feature which considerably enhances its value. In recent studies with a human virus the two distinct patterns which are seen for segments 10 and 11 appear to correlate with two major neutralization subgroups of the virus (4, 8). In addition, other major surveys of human viral genome electropherotypes have revealed considerable minor variations (12, 18), and one study has suggested that isolates from neonates may be distinct (18). Lourenco et al. (12) noted the limitation that their clinical samples were too small to allow multiple electrophoretic analyses. Silver staining should permit far more economical use of the dsRNA and has the added advantage

that the low loadings required enhance the resolution obtained. The adoption of the gel method for diagnosis should lead to a rapid increase in our understanding of rotavirus epidemiology. The method avoids the problems posed by the recent discoveries of rotaviruses without the group antigen (15, 19) and may also reveal whether the virus can cross species barriers as was recently suggested by McNulty et al. (15).

Most of the samples we have studied to date would be suitable for electropherotype analysis without further purification, but those which give high backgrounds could be conveniently purified by CF11 chromatography (1, 6). The gel system we describe above was selected to allow rapid staining of the gel after electrophoresis and not for maximum resolution of the dsRNA. Discontinuous buffer system gels (11) give the best resolution (18), but such gels require the full fixation and washing procedure described by Sammons et al. (20) to remove sodium dodecyl sulfate. Gels containing agarose cannot be stained by this method.

The method has several other minor advantages. The initial phenol extraction is both virucidal and bacteriocidal and thus eliminates the biohazard and much of the unpleasantness associated with fecal samples. The apparatus and chemicals employed are relatively inexpensive, and there is no dependence on immunological reagents which are variable and expensive to purchase or prepare.

The use of silver staining to detect nucleic acids in such low amounts should have considerable application in the study and diagnosis of other viruses, but it is particularly applicable to dsRNA because of the very sharp bands formed by this species in PAGE and because of the ease with which it may be purified from complex mixtures by CF11 cellulose chromatography (6). We have already found the method most useful for the detection of DNA in velocity sedimentation experiments and for the analysis of small restriction enzyme fragments.

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s required enhance the adoption of the gel could lead to a rapid finding of rotavirus epidemics the problems posed of rotaviruses without and may also reveal cross species barriers as McNulty et al. (15). We have studied to date electrophoretic analysis of rotavirus, but those which could be conveniently electrophoresed (1, 6). The gel was selected to allow after electrophoresis and detection of the dsRNA. Thin gels (11) give the thin gels require the full procedure described by move sodium dodecyl sulfate agarose cannot be

all other minor advantages extraction is both virus and thus eliminates the unpleasantness associated. The apparatus and relatively inexpensive, success on immunological methods is both simple and expensive to

ing to detect nucleic acids should have considerable study and diagnosis of particularly applicable to very sharp bands formed and because of the ease achieved from complex size chromatography (6). The method most useful in velocity sedimentation the analysis of small particles.

#### ACKNOWLEDGMENTS

F. Jamieson of the Edinburgh supplying the human fecal for their kind gift of Silane capable technical assistance, and the gels.

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## Detection of Subnanogram Amounts of RNA in Polyacrylamide Gels in the Presence and Absence of Protein by Staining with Silver

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Received March 8, 1982

The new ultrasensitive photochemically derived silver stain described for polypeptides in polyacrylamide gels (Merril *et al.*, *Science* 211, 1437-1438 (1981)) also stains nucleic acid in polyacrylamide gels. Reovirus genome double-stranded (ds) RNA segments were clearly detected in gels at about 0.03 ng/mm<sup>2</sup> with the silver staining technique when either purified virions or isolated, purified dsRNA was analyzed. The silver stain was about 10 to 30 times more sensitive than ethidium bromide for detecting reovirus dsRNA.

A highly sensitive silver stain for visualizing polypeptides in polyacrylamide gels has recently been developed by adapting methods of histology and photochemistry (1,2). The silver stain is about 100 times more sensitive than the conventional Coomassie brilliant blue stain for detecting polypeptides in gels and approaches the sensitivity of autoradiographic methods (1-4).

During the course of study on the interferon-induced protein kinase that is activated by double-stranded (ds)<sup>2</sup> RNA (5,6), we observed that the silver stain recently described for polypeptides (1,2) was also a highly sensitive stain for visualizing dsRNA in polyacrylamide gels. We believe that this observation may be useful to investigators studying the structure and function of protein-nucleic acid complexes such as virus particles and other nucleoprotein complexes.

### MATERIALS AND METHODS

**Materials.** Acrylamide was purchased from Eastman-Kodak and was recrystallized from chloroform prior to use; *N,N'*-methylenebisacrylamide was from Bio-Rad.

<sup>1</sup> To whom correspondence should be addressed.

<sup>2</sup> Abbreviation used: ds, double-stranded.

Sodium dodecyl sulfate (NaDodSO<sub>4</sub>) was obtained from Matheson, Coleman and Bell and was recrystallized from ethanol and washed with ether prior to use. *N,N,N',N'*-Tetramethylethylenediamine was from Sigma. Ethidium bromide was from Calbiochem, Coomassie brilliant blue R from Sigma or Serva, and methylene blue from Fisher or Sigma. All other chemicals were of reagent grade.

**Preparation of reovirions and reovirus double-stranded RNA.** The Dearing strain of reovirus type 3 was grown in mouse L fibroblasts in suspension culture, and reovirions were purified from infected cells by Freon extraction, velocity centrifugation on sucrose gradients, and equilibrium centrifugation on preformed cesium chloride gradients (7). Reovirus genome dsRNA was isolated from purified reovirions by repeated extraction with NaDodSO<sub>4</sub> and phenol, ethanol precipitation, and centrifugation (8). Virion concentrations were determined spectrophotometrically, with 5.42 OD units at 260 nm taken equivalent to 1 mg of virus (7). Protein concentrations were determined by a modification of the phenol reagent method of Lowry (9) with bovine serum albumin as the reference standard. RNA con-

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centrations were also determined spectrophotometrically, with 16.7 OD units at 260 nm taken equivalent to 1 mg of dsRNA (8).

**Electrophoresis.** Standard conditions for electrophoresis using a discontinuous Tris-glycine-buffered NaDodSO<sub>4</sub> polyacrylamide gel system were essentially as described by Laemmli (10) and modified by Studier (11). Slab gels made with 16 × 18-cm glass plates were 0.75 mm thick with a 5% acrylamide stacking layer and a 10% running layer. Virion and dsRNA samples were mixed with an equal volume of 2× sample extraction buffer containing NaDodSO<sub>4</sub> and mercaptoethanol and heated at 90°C for 2 min prior to use. Samples of 10 μl were loaded into wells 3.5 mm wide and electrophoresed at 25 to 30 mA for 2.5 to 3.0 h at ambient temperature.

**Staining.** (A) Silver: After electrophoresis, gels were fixed and stained with silver nitrate exactly as described by Merrill *et al.* (1). (B) Ethidium bromide and methylene blue: Before the gels were stained for nucleic acid with either ethidium bromide (12,13) or methylene blue (14), they were washed in glass-distilled, deionized (Nanopure, Barnstead) H<sub>2</sub>O to remove excess NaDodSO<sub>4</sub>. Gels were stained with 0.01% (w/v) ethidium bromide or methylene blue in 0.02 M sodium acetate for 2 h and were destained by washing in 0.02 M sodium acetate. (C) Coomassie brilliant blue: Before the gels were stained for protein with Coomassie brilliant blue R (15), they were fixed for 1 h in 25% (v/v) isopropanol-10% (w/v) trichloroacetic acid. Gels were stained with 0.1% (w/v) Coomassie brilliant blue in 25% isopropanol-10% trichloroacetic acid for 2 h and were destained by washing in 7% (v/v) acetic acid. Stained gels were photographed with a Polaroid MP-4 Land camera system using Polaroid 665 film.

## RESULTS AND DISCUSSION

The genome of mammalian reoviruses consists of 10 segments of double-stranded RNA which fall into three size classes: three

large (L) segments ( $M_r$  2.5–2.7 × 10<sup>6</sup>), three medium (M) segments ( $M_r$  1.2–1.4 × 10<sup>6</sup>), and four small (S) segments ( $M_r$  0.6–0.8 × 10<sup>6</sup>). These dsRNA segments code for a series of polypeptides that also fall into three size classes designated λ (large), μ (medium), and σ (small) (16,17). Reovirion particles are composed by mass of about 15% RNA and 85% protein (16,18). The 10 dsRNA segments, which are present in equimolar amounts in virions, account for approximately 75% of the RNA; the remaining 25% of the RNA is composed of small ssRNA oligonucleotides, which are most likely abortive transcription products (17).

The separation of various concentrations of purified reovirions on a NaDodSO<sub>4</sub>-polyacrylamide gel that was stained with the new photochemical silver stain described for polypeptides (1,2) is shown in Fig. 1. In addition to detecting the λ, μ, and σ virion capsid polypeptides, the new silver stain also detected the L, M, and S genome double-stranded RNA segments (Fig. 1). The silver stain appeared to be even more sensitive for reovirus dsRNA than for reovirus capsid polypeptides. The dsRNA segments were detectable by silver staining at virus dilutions where the capsid polypeptides were no longer visualized. Individual reovirus capsid polypeptides are present in different amounts in virions (17); σ<sub>3</sub> which represents 28% of the virion protein, σ<sub>2</sub> (7%), μ<sub>1c</sub> (35%), and μ<sub>1</sub> (2%) were well resolved from the dsRNA segments in the gel shown in Fig. 1. These viral polypeptides were routinely clearly detected with the silver stain at 2 ng protein/mm<sup>2</sup>; by contrast, the reovirus dsRNA segments were clearly detected with 0.03 ng dsRNA/mm<sup>2</sup>. For comparative purposes, the minimum concentration of bovine serum albumin detectable with the silver stain in polyacrylamide gels has been reported to be from 0.07 (3) to 0.38 (4) ng protein/mm<sup>2</sup>. The silver stain was about 50–100 times more sensitive than the Coomassie brilliant blue stain for detecting reovirus polypeptides in gels (data not shown).



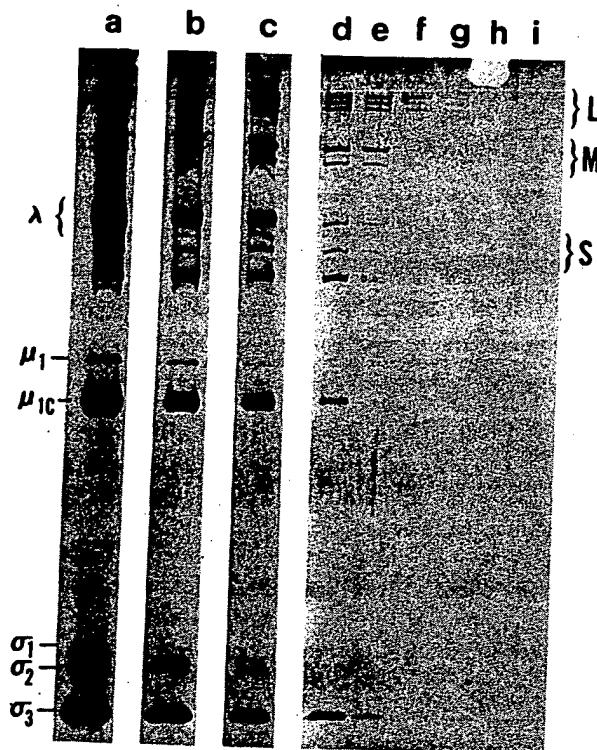


FIG. 1. Electrophoretic pattern of reovirion polypeptides and RNA stained with silver. Sample preparation, electrophoresis, and staining were as described under Materials and Methods. The following amounts of purified, disrupted virions (chemical composition: 85% protein, 15% RNA) were analyzed: (a) 10  $\mu$ g, (b) 3  $\mu$ g, (c) 1  $\mu$ g, (d) 300 ng, (e) 100 ng, (f) 30 ng, (g) 10 ng, (h) 3 ng, and (i) 1 ng of virion.  $\lambda$ ,  $\mu_1$ ,  $\mu_{1c}$ ,  $\sigma_1$ ,  $\sigma_2$ , and  $\sigma_3$  indicate the positions of capsid polypeptides; L, M, and S indicate the positions of genome dsRNA segments.

Ethidium bromide is the dye commonly used for staining double-stranded RNA (12) and DNA (13), although reovirus single-stranded messenger RNA (19) is also stained with ethidium bromide. As shown in Fig. 2, the silver stain (Fig. 2A) was about 10–30 times more sensitive than ethidium bromide (Fig. 2B) for detecting reovirus dsRNA in polyacrylamide gels. Reovirus genome dsRNA segments were clearly detected with the silver stain at about 0.03 ng dsRNA/ $\text{mm}^2$  (Figs. 1 and 2A), whereas with ethidium bromide only about 0.45 ng dsRNA/ $\text{mm}^2$  was detected (Fig. 2B).

The results of staining reovirus dsRNA with silver shown in Fig. 1 were obtained with purified virion particles that had been

disrupted with NaDodSO<sub>4</sub>, mercaptoethanol, and heat; the results shown in Fig. 2 were obtained with purified genome dsRNA isolated from virion particles. Although certain animal viruses such as adenovirus (20) and poliovirus (21) do contain protein linked to the 5' ends of their genomes, reovirus dsRNA does not contain protein covalently linked to the ends of the genome dsRNA segments, as demonstrated by direct sequence analysis of some individual segments (17). Furthermore, treatment of purified reovirus dsRNA with proteinase K (1 mg/ml for 3 h) did not alter the ability of silver to stain the dsRNA segments when separated by polyacrylamide gel electrophoresis (data not shown). These results suggest that

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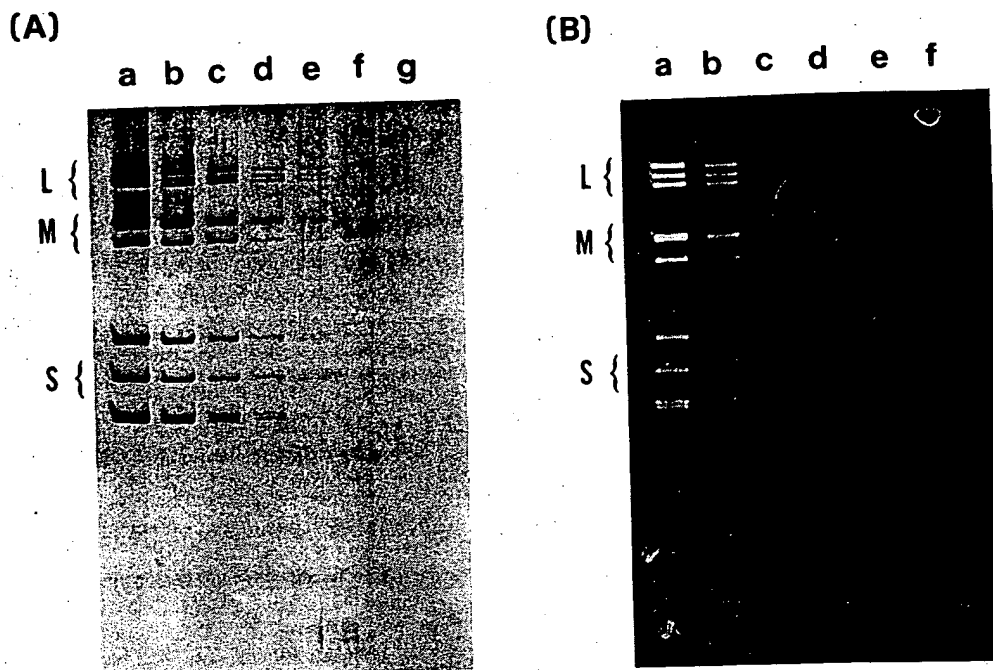


FIG. 2. Comparison of techniques for staining purified reovirus genome double-stranded RNA. The following amounts of reovirus dsRNA isolated from purified virion particles were analyzed: (a) 100, (b) 30, (c) 10, (d) 3, (e) 1, (f) 0.3, and (g) 0.1 ng of dsRNA. (A) Gel was stained with the photochemical silver technique and photographed under visible light; (B) gel was stained with ethidium bromide and photographed under ultraviolet light. Conditions were as described under Materials and Methods; L, M, and S indicate the three size classes of the dsRNA segments.

silver stains dsRNA both in the absence (Fig. 2) and presence (Fig. 1) of polypeptide components in the sample mixture separated by electrophoresis. We have also detected dsDNA in agarose gels with the silver stain, although variable and high background staining was experienced with agarose gels (data not shown). During the preparation of our work for publication, we became aware of the recent results of Somerville and Wang (22), who showed that *Escherichia coli* ribosomal RNA and bacteriophage  $\lambda$  DNA were stained by silver in polyacrylamide gels.

In addition to mammalian reoviruses, there are numerous other viruses of vertebrates, insects, and plants that possess double-stranded RNA genomes (16,17). Some of these viruses are of agricultural and medical importance, and some are relatively dif-

ficult to grow in culture or under conditions where the genome can be conveniently labeled to high specific activity with radioactive precursors. The fact that subnanogram amounts of dsRNA can be detected in polyacrylamide gels with the photochemical silver stain may be useful in biochemical and genetic studies where limited amounts of dsRNA are available for characterization.

#### ACKNOWLEDGMENTS

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TRANSLATION OF AVIAN SARCOMA VIRUS RNA IN XENOPUS LAEVIS OOCYTESRichard A. Katz<sup>1</sup>, George M. Maniatis<sup>2</sup>, and Ramareddy V. Guntaka<sup>1</sup>Departments of Microbiology<sup>1</sup> and Human Genetics<sup>2</sup>,  
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Received November 16, 1978

Summary: Evidence for the synthesis and processing of Pr76 (precursor to group-specific antigens p27, p19, and p12/15, upon injection of avian sarcoma virus 70S or 35S RNA into Xenopus oocytes has been presented. Further, we show that tRNA<sup>trp</sup> primer, bound to 35S RNA, does not block translation of virion RNA under these conditions.

The 35S virion and 35S messenger RNA's of avian sarcoma virus contain four genes, which have been ordered as 5' gag-pol-env-src-3'

(1). The gag gene codes for a protein of 76,000 daltons (Pr76), which is subsequently processed to give rise to the major internal virion proteins (p27, p19, p15, and p12), the so-called gs antigens (2). The characteristics of these and other virus proteins have been described

(2). Several groups have independently translated 35S RNA in in vitro systems such as Krebs ascites and reticulocyte lysates (3-6). The limitation of most cell-free translation systems, however, appears to be the absence of post-translational cleavage of precursor molecules to give rise to appropriate final products.

Several species of tRNAs are associated with the 60-70S complex of virion RNA. One of these which has been shown to be specific for tryptophan (tRNA<sup>trp</sup>), is located near the 5' end of 35S RNA subunit, and functions as a primer for DNA synthesis (7). In avian sarcoma virus-infected cells the 35S RNA serves as a messenger and also is encapsidated in the virion. In an effort to investigate the regulation of 35S by bound tRNA<sup>trp</sup> primer of avian sarcoma virus, we have decided to use the Xenopus laevis oocyte translation system. The major advantages of this system, as opposed to the cell

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free systems are: 1) the requirement for relatively small quantities of exogenous mRNA and 2) the injected mRNAs are translated for longer periods of time (8).

In this brief communication we will describe our results which show 1) the synthesis and post-translational processing of Pr76 after micro-injection of avian sarcoma virus 35S subunits or aggregated 60-70S virion RNA, confirming earlier work on avian myeloblastosis virus RNA (9) and 2) that bound tRNA<sup>trp</sup> primer does not appear to affect the capacity of 35S virion RNA to direct protein synthesis.

#### MATERIALS AND METHODS

Cells and Viruses: Avian sarcoma virus, (ASV) Prague C, was propagated in chick embryo fibroblasts (CEF) (10). The cells prepared from 11 to 12 day-old embryos (Spafas, Connecticut), were grown in medium 199 as previously described (11).

Immunoprecipitation and Gel Electrophoresis of Viral Proteins: Anti-viral anti-serum was raised in New Zealand white rabbits essentially as described previously (12). Virion 70S RNA was prepared from ASV-PrC or PrB virus and it was resuspended at a concentration of 250 µg in oocyte injection buffer (88 mM NaCl, 1 mM KCl, 15 mM Tris-HCl pH 7.6). *X. laevis* frogs were obtained from Charles W. Fletcher, Hampstead, Md. For each RNA sample 10-12 oocytes at stage V-VI (8) were injected. Oocytes were incubated at 22° for the periods indicated below in 100 µl modified Barth's solution (13) containing 100-150 µCi <sup>35</sup>S-methionine (300 Ci/mole). <sup>35</sup>S-methionine labeled PrB virus was prepared as previously described (2). For analysis of intracellular viral proteins, PrB virus-infected chick embryo fibroblasts were labeled with 100 µCi/ml <sup>35</sup>S-methionine in methionine-free Eagle's medium containing 10% dialyzed calf serum. Immunoprecipitation was carried out according to Kessler using Cowan I strain *Staphylococcus aureus* as an immune absorbant (14). Oocytes and cells were washed in Barth's medium and PBS respectively and lysed in 0.5-1 ml NET (0.1 M NaCl, 5 mM EDTA, 20 mM Tris-HCl pH 7.4) containing 0.5% NP-40. Cellular debris was removed by centrifugation. All samples were first treated with 5-15 µl of preimmune serum, followed by precipitation with 2-5 µl of immune serum for 30 minutes at 40C, using the bacterial absorbant for both steps. After absorption, bacteria were washed 3 times in 0.05% NP-40 NET buffer, resuspended in 10 µl of SDS-gel sample buffer (3% SDS, 0.01% phenol red, 1% mercaptoethanol, 50 mM Tris-HCl pH 6.8, 10% glycerol) and boiled for 3 minutes. Bacteria were removed by centrifugation and the resulting supernatants were analyzed on 17.5% SDS-acrylamide gels (15). The <sup>35</sup>S-labeled protein bands were located by fluorography (16).

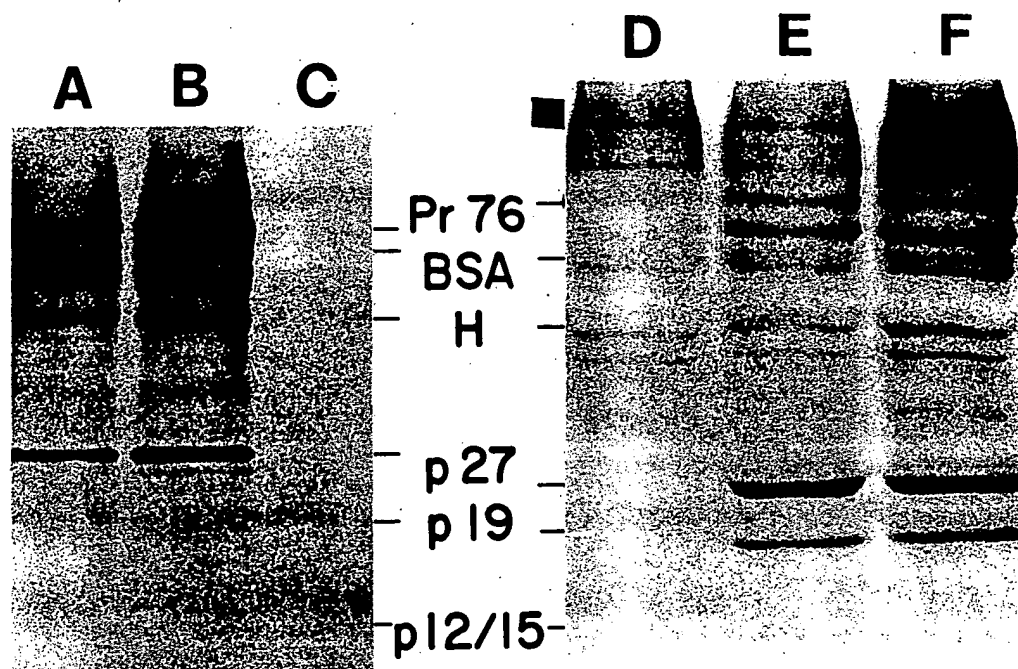
Assay for the Release of tRNA Primer: tRNA<sup>trp</sup> primer which is bound to the virion RNA, was specifically labeled with <sup>32</sup>P by initiating complementary DNA synthesis in the presence of dCTP analogue, Ara-CTP, essentially as described by Taylor et al. (17). The reaction mixture containing 50 mM Tris-HCl pH 7.4, 50 mM KCl, 10 mM dithiothreitol, 10 mM MgCl<sub>2</sub>, 0.05% NP-40, 10 µM dGTP, dTTP

(P-L Biochemicals),  $\beta$ -D-furanosyl arabinoside triphosphate (Ara CTP), 600  $\mu$ Ci/ml  $\{\alpha\text{-}^{32}\text{P}\}$  dATP (NEN) and purified avian sarcoma virus, was incubated at 37°C for 1 hour. The 70S RNA- $^{32}\text{P}$ -cDNA product was separated from the free isotope on sucrose gradient, extracted with phenol, and concentrated by ethanol precipitation. The complexes were resuspended in oocyte injection buffer and heated to the temperatures as indicated in Figure 2. Release of the  $\{\text{}^{32}\text{P}\}$  cDNA-tRNA covalent complex from the 70S RNA was monitored by electrophoresis in 10% acrylamide gels using a tris-borate buffer system.  $\{\text{}^3\text{H}\}$  *E. coli* tRNA was included in each sample as an internal marker.

## RESULTS AND DISCUSSION

*Xenopus laevis* oocytes were injected with avian sarcoma virus RNA (250  $\mu$ g/ml, 50 ml/oocyte) from Prague C (PrC) as previously described (18). The oocytes were incubated with  $^{35}\text{S}$ -L-methionine-containing medium for 5, 24, or 48 hours. The proteins were isolated from oocytes and radio-labeled proteins were immunoprecipitated with rabbit antiserum directed against Triton X-100 disrupted virions. The immune complexes were separated by adsorption to formalin-fixed *S. aureus* cells (14) and then analyzed by polyacrylamide gel electrophoresis.

The results presented in Figure 1 show that virus specific proteins can be translated in oocytes in response to injected viral RNA, as evidenced by the size of the proteins which are precipitated by immune serum. No bands corresponding to viral proteins are visible in samples treated with pre-immune serum (data not shown). There are no immunoprecipitable proteins from uninjected controls (Fig. 1c and d). Five hours after injection the major band correspond to Pr76 (data not shown). In addition to pr 76, p27, two other high molecular weight viral proteins with molecular weights of approximately 64,000 and 58,000 daltons have appeared after 24 hours (Fig. 1a-c). This cleavage pattern is similar to that seen in infected cells (2). Analysis of 48 hour-labeled oocyte lysates revealed additional bands corresponding to viral proteins p19 and p15/12 (Figure 1, e and f). (Protein p15/12 appears faintly in the photograph, but is clearly visible on the autoradiogram). The band labeled H results from non-specific trapping by immunoglobulin heavy chains.



**Figure 1.** Autoradiogram of SDS-polyacrylamide gel electrophoresis of  $^{35}\text{S}$ -methionine labeled proteins precipitated from *X. laevis* oocyte lysates. a-c, oocytes incubated for 24 hours. Viral RNA heated for 3 minutes at (a)  $100^{\circ}\text{C}$ , (b)  $50^{\circ}\text{C}$  prior to injection; (c) uninjected control; d-f, oocytes incubated for 48 hours; (d) uninjected or viral RNA heated (e)  $50^{\circ}\text{C}$  or  $80^{\circ}\text{C}$  (f) prior to injection.

These results support the notion that the group-specific proteins (gag gene products) are coded by 35S RNA (3,4,9). That the oocyte system, in contrast to the cell-free systems, has the ability to process precursor proteins into the expected final products is also evident from these data.

Previous studies from other laboratories indicated that the  $T_m$  for dissociating bound  $\text{tRNA}^{\text{Phe}}$  from 35S RNA is  $63^{\circ}\text{C}$  in 0.01 M Tris-HCl-0.01 M EDTA (18). ASV RNA heated at  $50^{\circ}\text{C}$ ,  $80^{\circ}\text{C}$  or  $100^{\circ}\text{C}$  in 88 mM NaCl buffer prior to injection did not reveal any major differences in its ability to direct protein synthesis although structural changes in the RNA molecule were evident. We found, in agreement with published results (19), that approximately 30% of the 60-70S RNA dissociated into 35S RNA subunits at  $50^{\circ}\text{C}$  and  $80^{\circ}\text{C}$  all the RNA sedimented at 35S. Since the injection buffer contains 88 mM NaCl the following



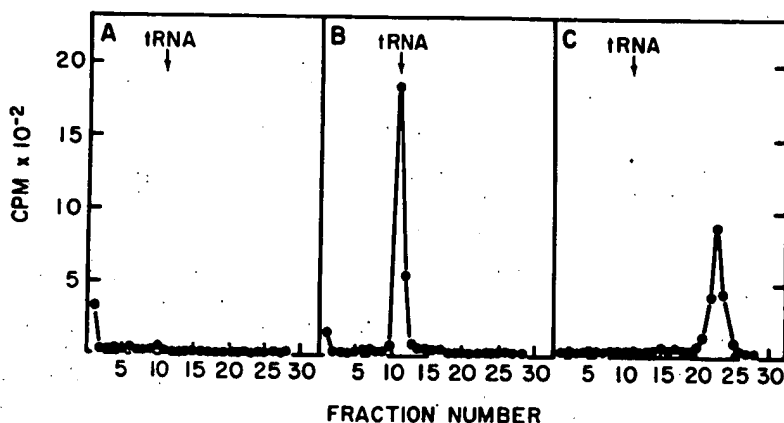


Figure 2. Polyacrylamide gel assay for the release of primer. (a) 60-70S RNA containing <sup>32</sup>P-labeled octamer-tagged primer run as a control; (b) complex heated at 80°C in 88 mM NaCl and (c) complex digested with 0.2 M NaOH at 100°C for 3 minutes prior to analyzing on the gel.

experiment was performed to prove that under the conditions of melting all the tRNA primer was removed from 60-70S RNA. Purified ASV was incubated in an endogenous reaction containing <sup>32</sup>P labeled dATP as described in the Methods section and ara-CTP replaced dCTP. In the presence of this analogue the major product is an oligo-nucleotide of 8 residues (octamer) covalently linked to the primer (16). After the reaction, viral RNA containing the radio-labeled primer was isolated, heated at 80°C in 88 mM NaCl, and analyzed in polyacrylamide gels. Under these conditions the tRNA primer which is tagged with <sup>32</sup>P-labeled octamer was completely dissociated from 60-70S RNA as evidenced by a discrete peak of radioactivity comigrating with *E. coli* tRNA marker (Fig. 2b). In the unheated control the radio-activity remained at the origin indicating that the primer is associated with 60-70S RNA (Fig. 2a). Digestion of the product with alkali, which removes the RNA molecule of the hybrid, caused the DNA to migrate at the expected position for an octamer (Fig. 2c). From these results we conclude that the conditions used to release the primer are satisfactory. Injection of the RNA samples heated at these temperatures did not reveal any major variations in the levels of translation (Fig. 1). However we

observed that RNA heated at 50°C was sometimes better in its capacity to translate than that heated at more than 80°C. This is probably due to random breaks introduced in the RNA molecule at higher temperatures. In addition, we consistently found that RNA which was not heated had translation ability similar to heated samples (data not shown).

These results indicate that under the conditions used here for translation, virion 35S RNA as well as the 60-70S complex can be used as a template for the synthesis of viral proteins. In addition, the data presented here suggest, but do not prove, that initiation of protein synthesis is internal to the primer binding sequence, since the presence of primer has no apparent effect on translation\*. If this is true, then the results may mean that the primer has no role in distinguishing 35S mRNA from 35S virion RNA. In support of this argument data presented by Canaani and Duesberg (20), indicated that the RNA from immature virions does not contain bound primer, but, after incubation at 37°C, complete 60S-70S-primer complex can be generated.

#### ACKNOWLEDGEMENTS

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\* In agreement with these data, amino acid sequence data indicated that the possible initiation AUG codon beginning at nucleotide residue 83 (which is on the 5' end of tRNA<sup>trp</sup> binding site) does not serve as an initiation codon for Pr76 (R. Eisenman, personal communication).

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PHYSICAL CHEMISTRY AND BIOPHYSICS

# Research Communications



# Translation of MuLV and MSV RNAs in Nuclease-Treated Reticulocyte Extracts: Enhancement of the gag-pol Polypeptide with Yeast Suppressor tRNA

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## Summary

The virion RNAs from Moloney murine leukemia virus (MuLV) and Moloney murine sarcoma virus (MSV) were translated in a micrococcal nuclease-treated cell-free system from rabbit reticulocytes. The predominant polypeptides formed from 35S MuLV RNA were 78,000 and 65,000 daltons in molecular weight, and minor components with molecular weights of 180,000, 110,000, 52,000 and 40,000 daltons were also observed. The 30S MSV RNA yielded two predominant polypeptides of 62,000 and 43,000 daltons, and minor components about 72,000, 40,000 and 18,000 daltons in molecular weight. The predominant polypeptides generated by both MuLV and MSV RNA were found to be precursors of the core proteins by immunoprecipitation with specific antisera. The 180,000 dalton molecular weight polypeptide encoded by MuLV RNA was immunoprecipitated both by antisera to the core protein (p30) and reverse transcriptase. The major products therefore appear to be Pr65<sup>gag</sup> and Pr78<sup>gag</sup>; an important minor product is Pr180<sup>gag-pol</sup>. Most of the products of <65,000 daltons synthesized in either system contained sequences from the core protein precursor, but the 43,000 and 18,000 dalton molecular weight polypeptides generated by MSV RNA did not precipitate with antisera to MuLV proteins.

When purified yeast suppressor tRNA was added to the translation mixture directed by 35S MuLV RNA, the amount of the Pr78<sup>gag</sup> was reduced, while the yield of the Pr180<sup>gag-pol</sup> was enhanced. Amber suppressor tRNA was about 3 times as effective as ochre suppressor tRNA and nonsuppressor tRNA. This pattern of suppression was also seen for an established amber mutation (UAG) in the synthetase gene of Q $\beta$  (Q $\beta$  aml), suggesting that it is a UAG codon which terminates synthesis of Pr78<sup>gag</sup>. In the MSV system, the amber suppressor tRNA, and to a lesser

extent the ochre suppressor tRNA, markedly increased the synthesis of the 72,000 dalton molecular weight polypeptide with a slight reduction of the 62,000 dalton protein.

Since read-through between the core protein and reverse transcriptase genes occurs to a low level both in vivo and in vitro and can be enhanced in vitro by amber suppressor tRNA, these results suggest that a suppression mechanism may control the relative amounts of core protein and reverse transcriptase synthesized from 35S mRNA. Such a mechanism might be used more generally by mRNAs from mammalian cells.

## Introduction

The synthesis of functional retrovirus proteins involves a complicated series of processing events. There are three known genetic regions present in the genomes of nondefective retroviruses: *gag*, the region encoding the internal virion structural proteins; *pol*, the reverse transcriptase gene; and *env*, the region encoding the glycoproteins of the virion envelope outer surface (Baltimore, 1974). In the avian leukosis viral RNA, these regions are apparently located on the RNA genome in the order (5'  $\rightarrow$  3') *gag-pol-env* (Joho et al., 1976; Wang et al., 1976), although the order of the first two genes is still somewhat ambiguous. Presumably, other retrovirus genomes have the same gene order.

There are at least two mRNAs that are used for translation of both murine and avian viral proteins. One is thus far indistinguishable in size from virion RNA, while the other—a much smaller RNA—appears to encode only the *env* region (Hayward, 1977; Pawson, Harvey and Smith, 1977; Stacey, Allfrey and Hanafusa, 1977; Van Zaane et al., 1977; Weiss, Varmus and Bishop, 1977). Each of the three genetic regions encode precursors to the functional proteins; the precursors are proteolytically processed to generate the final products (Vogt and Eisenman, 1973; Jamjoom, Naso and Arlinghaus, 1977).

One of the most intriguing aspects of the multi-layered scheme of gene expression used by retroviruses is synthesis of the reverse transcriptase. Studies of cells infected with avian or murine viruses have given the same general results, except that the molecular weights of the relevant polypeptides are somewhat different; we will use the sizes of the murine proteins for this discussion. (The nomenclature of the proteins will follow the recommendations of the NCI Tumor Viral Immunology Workshop, March, 1977.) Available evidence on the synthesis of the reverse transcriptase (p85) is first, that no discrete mRNA for synthesis of the

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enzyme has been detected; rather 35S mRNA appears to encode both Pr78<sup>gag</sup> (the precursor of the structural proteins of the virus with a major partial product of Pr65<sup>gag</sup>) and Pr180<sup>gag-pol</sup> (a polypeptide containing antigens of both reverse transcriptase and Pr78<sup>gag</sup>) (Naso et al., 1975; Mueller-Lantzsch and Fan, 1976; Kerr et al., 1976; Oppermann et al., 1977). Second, the molarity of reverse transcriptase made in infected cells is 0.1–0.01 of the molarity of virion structural proteins (Panet, Baltimore and Hanafusa, 1975; Jamjoom et al., 1977). Third, by enzyme assay, there is little or no detectable soluble reverse transcriptase in infected cells; the enzyme is apparently activated only upon incorporation into virions (Panet et al., 1975). Fourth, Pr180<sup>gag-pol</sup> is probably cleaved to form the p85 reverse transcriptase inside maturing or mature virions (Jamjoom et al., 1977; Oppermann et al., 1977; O. N. Witte and D. Baltimore, unpublished observations). Fifth, the cleavage of Pr180<sup>gag-pol</sup> to p85 appears to involve intermediates that have no gag protein determinants, the main one being Pr110<sup>pol</sup> (Jamjoom et al., 1977). Sixth, only Pr180<sup>gag-pol</sup> but no Pr110<sup>pol</sup> or p85 is evident in cells infected under restrictive conditions by certain temperature-sensitive murine leukemia viruses, but both appear after shift to permissive conditions, indicating that Pr180<sup>gag-pol</sup> gives rise to both Pr110<sup>pol</sup> and p85 (O. N. Witte and D. Baltimore, unpublished results).

These facts and interpretations leave open one critical question: what controls whether a ribosome, having initiated translation of 35S mRNA, will generate Pr78<sup>gag</sup> or—by read-through of the end of the gag-coding region—will generate Pr180<sup>gag-pol</sup>? One possibility is that a codon specifying termination of protein synthesis (amber, UAG; ochre, UAA; or UGA) follows the coding region for Pr78<sup>gag</sup>, but that a mechanism allows for occasional read-through of this termination codon (suppression). Such a model would be similar to the mechanism of synthesis of the read-through protein of Q $\beta$  phage (Weber and Konigsberg, 1975). To test the possibility of a suppressible codon allowing read-through, a cell-free translation system programmed by virion RNA of MuLV was used.

The cell-free translation of MuLV virion RNA in various systems has already been described. The major products made in extracts of tissue culture cells have been identified as Pr78<sup>gag</sup> (called 70K previously), Pr65<sup>gag</sup> (called 60K previously) and a 180,000 dalton polypeptide with at least gag protein determinants (Kerr et al., 1976). For the present studies, we have adopted the micrococcal nuclease-treated reticulocyte lysate system of Pelham and Jackson (1976) and have first described the products made by that system before investi-

gating the effect of yeast suppressor tRNAs.

Yeast suppressor tRNAs have been characterized that are capable of suppressing termination of amber and ochre codons (Hawthorne and Leupold, 1974; Cappechi, Hughes and Wahl, 1975; Gesteland et al., 1976). The suppressor tRNAs used in our studies were serine tRNAs modified by mutation to enable reading of UAG and UAA. They were characterized by their ability to suppress termination of translation of RNA from appropriate mutants of bacteriophage Q $\beta$  (Gesteland et al., 1976) and subsequently used by Gesteland et al. (1977) to identify nonsense mutants of an adeno-SV40 hybrid virus. In our studies, they have been used in a partially purified form as probes for the existence of UAG or UAA codons at the gag/pol junction. Suppression has been found at the gag/pol junction, and its pattern suggests that a UAG codon may be present there.

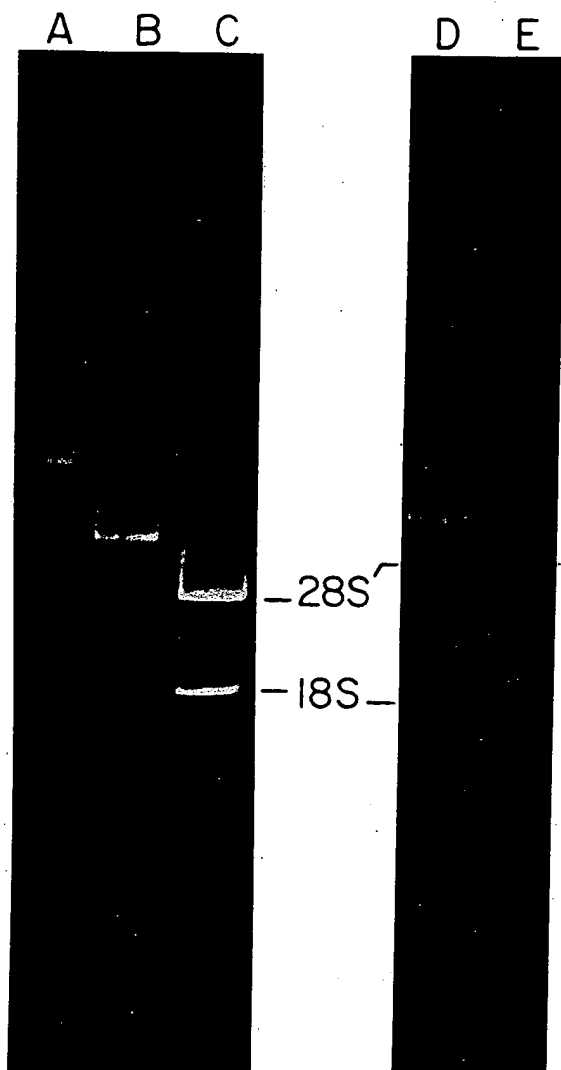
We have also extended these studies to investigate translation of the murine sarcoma virus (MSV) genome. Pure 30S MSV RNA was obtained from virions made by cells that produce almost exclusively MSV [TB(CFW/D) clone 124; Ball, McCarter and Sunderland, 1973]. Heteroduplex and hybridization analysis of this RNA has indicated that it is a highly deleted and substituted form of MuLV RNA (Frankel, Neubauer and Fischinger, 1976; Hu, Davidson and Verma, 1977). The only region on this RNA known to encode protein is one that makes P60 protein, a polypeptide with MuLV gag protein antigenicity (Robey et al., 1977).

## Results

### RNAs Studied

The translation studies described here used the virion RNAs from murine leukemia and sarcoma viruses (MuLV and MSV) as mRNAs. MuLV 70S RNA and MSV 50S RNA were denatured and fractionated on sucrose gradients to isolate the resulting 35S MuLV RNA and 30S MSV RNA. The undenatured RNAs were <10% as active as the denatured forms.

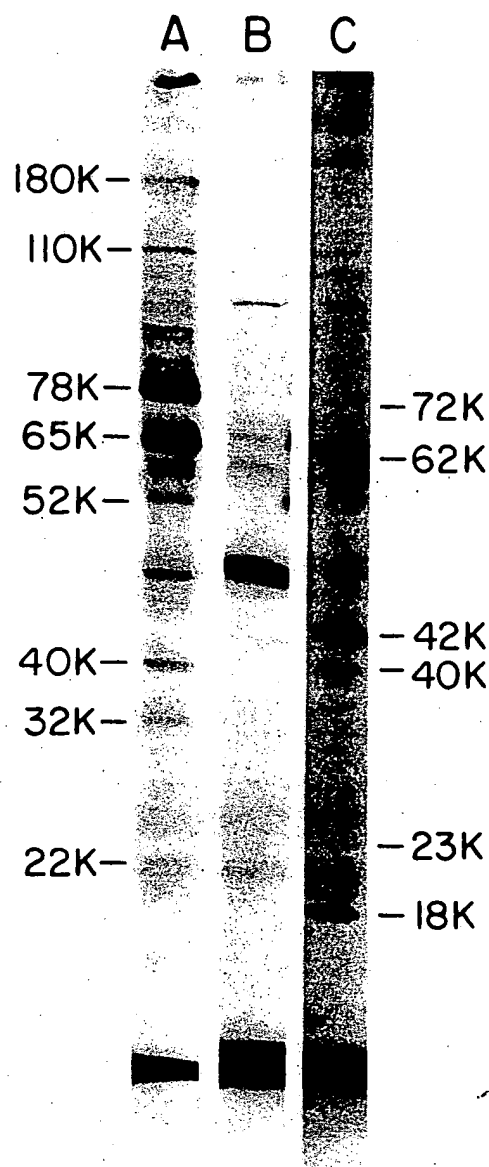
The purity and size of RNA preparations were established by gel electrophoresis. In a representative preparation, the majority of the MuLV RNA and MSV RNA migrated through 1% agarose gels as single bands with minor amounts of smaller RNA also evident (Figure 1, slots A and B); the major bands were slower in mobility than ribosomal RNA (slot C). The sizes of the majority RNA species were determined after electrophoresis through methylmercuric hydroxide-1% agarose gels (Figure 1, slots C and D). From these analyses, the molecular weights were estimated as 9.7 kilobases (MuLV RNA) and 6.5 kilobases (MSV RNA).



**Figure 1. Electrophoretic Analysis of Viral RNA**  
MuLV 35S RNA (A) and 30S MSV RNA (B) were analyzed together with ribosomal RNA (C) on a 1% agarose slab gel in 60 mM Tris-acetate (pH 7.2), 1 mM EDTA (Sharp et al., 1973). Electrophoresis was for 2 hr at 5 V/cm, and the RNA was stained with ethidium bromide. An artificial mixture of 30S MSV RNA and 35S MuLV RNA was analyzed on methylmercuric hydroxide gels (Bailey and Davidson, 1976) in the same slot (D) together with 35S MuLV RNA (E) in a separate slot. The ribosomal markers analyzed on the same gel are indicated by the arrows. The samples had 0.5  $\mu$ g of each RNA studied. Staining with ethidium bromide was carried out in 0.5 M  $\text{NH}_4$  acetate.

#### Cell-Free Synthesis

The micrococcal nuclease-treated rabbit reticulocyte lysate (Pelham and Jackson, 1976) was used for cell-free protein synthesis in all the present studies. To compare this system with others used previously, the protein products synthesized under the direction of MuLV and MSV RNA were characterized by electrophoretic separation (Figure 2). Without added RNA (slot B), faint bands of 96,000



**Figure 2. Translation of Murine Leukemia and Sarcoma Virus RNA in a Cell-Free System**

MuLV RNA (A) and MSV RNA (C) were translated in a micrococcal nuclease-treated reticulocyte lysate as described in Experimental Procedures. A control without added mRNA is included for comparison (B). The molecular weights of the polypeptides induced by the viral RNA are indicated as the molecular weight  $\times 10^{-3}$  (K).

(96K), 60-64K and a diffuse one at about 20K daltons were observed as well as a band of variable intensity at 47K daltons. The apparent band of 47K daltons is probably  $^{35}\text{S}$ -met-tRNA because it can be abolished by RNAase A treatment (not shown).

The MuLV RNA-specified product had two main bands of 78K and 65K daltons (slot A), the same

products found in other cell-free systems (Naso et al., 1975; Kerr et al., 1976). Additional bands of 180K, 110K, 52K, 40K, 32K and 22K daltons were also observed.

#### Identification of Polypeptides

For identification of these products, immunoprecipitation with defined antisera was used. The proteins used to immunize rabbits were highly purified p30, gp70 and p85 (reverse transcriptase), as shown in Figure 3 (slots A, B and C). The p30 preparation contained dimers as well as monomers. The specificity of each serum was tested by immunoprecipitation of <sup>35</sup>S-methionine-labeled MuLV virion proteins (Figure 3, slot D). The anti-p30 serum precipitated both p30 and the Pr65 found in virions (Figure 3, slot E). The anti-gp70 precipitated gp70 and p15E, two proteins which occur as a disulfide-linked complex in virions (Figure 3, slot F; Witte, Tsukamoto-Adey and Weissman, 1977). The anti-reverse transcriptase precipitated mainly p85 as well as a lower molecular weight product not further identified (Figure 3, slot G). Some nonspecific precipitation is evident with all the sera.

These antisera were used to characterize the products of cell-free synthesis directed by MuLV

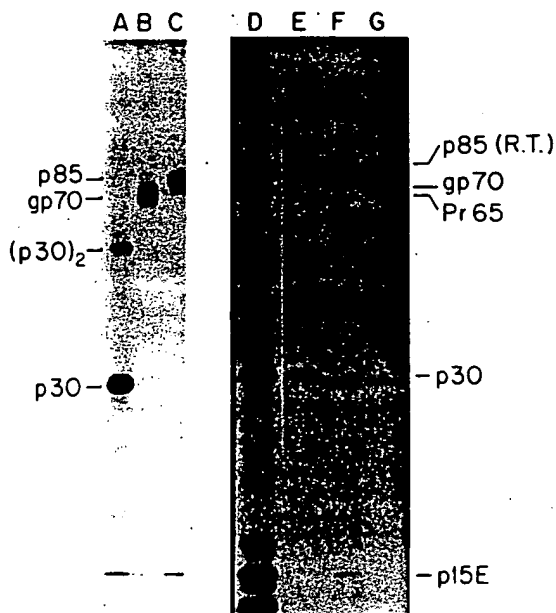


Figure 3. The Antigens Used for Immunization and the Specificity of the Resulting Sera

The proteins purified as described in Experimental Procedures were analyzed by electrophoresis (Laemmli, 1970). (A) p30 (and a dimer of p30); (B) gp70; (C) p85 (reverse transcriptase). To test the specificity of the sera, <sup>35</sup>S-methionine-labeled Moloney MuLV was reacted with each serum, and the precipitated proteins (see Experimental Procedures) were analyzed by electrophoresis. (D) the total MuLV proteins; (E) anti-p30; (F) anti-gp70; (G) anti-reverse transcriptase.

RNA. From the total MuLV RNA-specified product (Figure 4, slot A), most of the polypeptides were precipitated by anti-p30 antibody (Figure 4, slot B), and almost all of this precipitation could be competed by addition of unlabeled, purified p30 from MuLV virions (Figure 4, slot C) but not by purified reverse transcriptase (Figure 4, slot D).

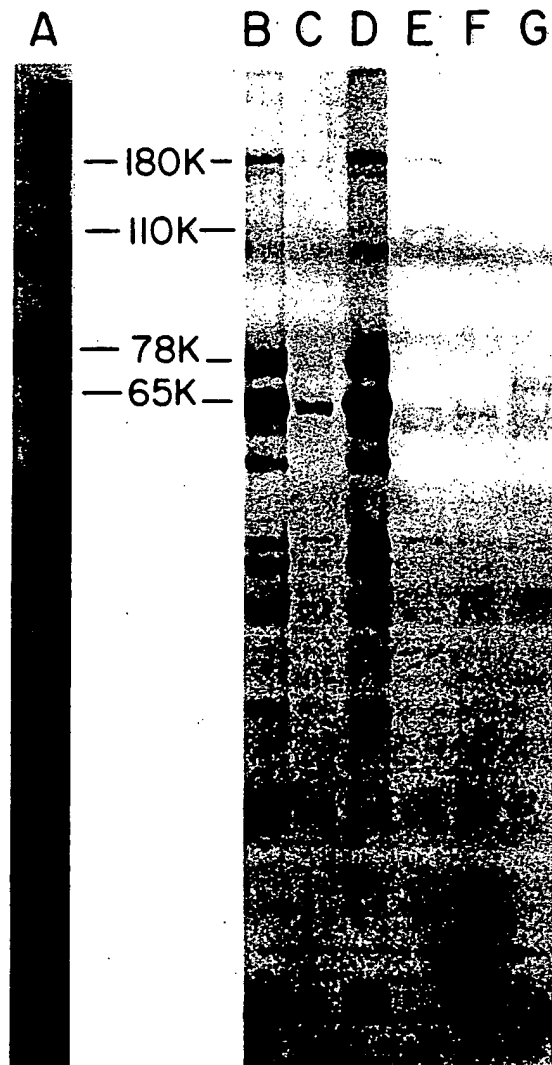


Figure 4. Immunoprecipitation of Polypeptides Generated by MuLV RNA in the Cell-Free System

Translation mixtures with MuLV RNA were immunoprecipitated with antibodies to the structural proteins of MuLV virus. (A) Polypeptides generated by MuLV RNA; (B) immunoprecipitate with anti-p30 serum; (C) immunoprecipitation with anti-p30 serum in the presence of an excess of purified p30; (D) immunoprecipitation with anti-p30 serum in the presence of an excess of purified reverse transcriptase; (E) immunoprecipitation with anti-reverse transcriptase serum in the presence of an excess of purified p30; (F) immunoprecipitation with anti-reverse transcriptase serum in the presence of excess of reverse transcriptase and p30 protein; (G) immunoprecipitation with anti-glycoprotein serum in an excess of purified p30 protein. The molecular weights of the polypeptides are indicated as in Figure 2.



The only polypeptides not well precipitated by anti-p30 serum were 110K and the minor bands between 110K and 78K.

By contrast, an anti-reverse transcriptase antibody, acting in the presence of added p30, precipitated 180K and 110K quite effectively (Figure 4, slot E); 80% of this precipitation was competed by purified reverse transcriptase (slot F). Quantitation was performed by scanning the autoradiograms.

An antiserum to the envelope glycoprotein selectively precipitated a 70K polypeptide (Figure 4, slot G). This polypeptide was not observed in the original mixed translation product and must therefore be synthesized in minute amounts. It appears to correspond to the size of the nonglycosylated glycoprotein of MuLV RNA (Van Zaane et al., 1977), but has not been characterized further.

This analysis of the products made under the direction of 35S MuLV virion RNA, coupled with earlier observations on other cell-free systems (Kerr et al., 1976), suggests that two major classes of products are formed: those with p30 antigenicity and those with both p30 and reverse transcriptase antigenicity. The 110K polypeptide may have only reverse transcriptase antigenicity. The p30 protein is representative of the products of the *gag* genes; thus precursors of the *gag* proteins can be described as Pr<sup>gag</sup>. The Pr65<sup>gag</sup> and Pr78<sup>gag</sup> are apparently direct products of translation from the most accessible initiation codon which is presumably near the 5' end of the MuLV RNA. The Pr180<sup>gag-pol</sup> appears to be an elongation of the *gag* precursor into the reverse transcriptase gene, producing a 180,000 dalton polypeptide. Analysis of infected cells has shown both the existence of Pr180<sup>gag-pol</sup> and its cleavage to form a reverse transcriptase precursor of 110,000 daltons (Jamjoom et al., 1977; their estimate of molecular weight was higher, but we believe that 110,000 daltons is closer to correct). The 110K described here has the same immunological properties as the in vivo 110K protein, so we tentatively designate it Pr110<sup>pol</sup>, but the identity of the in vivo and in vitro 110K products remains to be established.

Thus MuLV RNA appears to specify Pr<sup>gag</sup> and Pr<sup>gag-pol</sup> in the cell-free system at a ratio estimated as 50:1 (densitometry data not presented); control of this ratio appears to be determined by the frequency of read-through of the termination signal between the *gag* region and the *pol* region. We next investigated the nature of this termination codon.

#### Effect of Suppressor tRNAs on Translation of MuLV RNA

One well established mechanism for controlling translational read-through utilizes the ability of suppressor tRNAs to read a termination codon as

an amino acid (Gorini, 1970). The only eucaryotic suppressor tRNAs to be characterized to date are those from yeast (Capecchi et al., 1975; Gesteland et al., 1976). We therefore tested the ability of yeast suppressor tRNAs to increase the yield of Pr180<sup>gag-pol</sup> as an indication of the existence of a suppressible termination codon between *gag* and *pol* which could allow the rare read-through from *gag* into *pol*.

In our first attempts, we used crude tRNA from yeast suppressor strains having modified serine tRNAs able to read UAG (amber) or UAA (ochre) codons. Although Gesteland et al. (1977) had found that an amber codon in a mutated adeno-SV40 hybrid virus could be suppressed by such crude tRNA, we found only inhibition of synthesis directed by MuLV RNA (not shown), and we therefore used only fractionated tRNA in further studies.

When a cell-free system programmed by MuLV RNA—which gave the usual faint bands at 110K–180K daltons (Figure 5, slot A)—was supplemented with a purified yeast amber suppressor tRNA, a marked increase of Pr180<sup>gag-pol</sup> was observed along with increased amounts of bands from 110K–180K daltons (Figure 5, slot D). A concomitant loss of radioactivity in the Pr78<sup>gag</sup> band was also produced

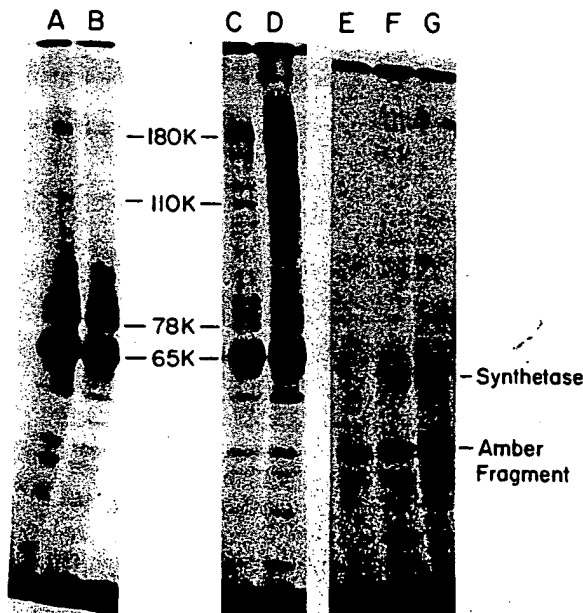


Figure 5. Yeast Suppressor tRNA Causes Read-through of the *gag-pol* Genes of the MuLV Genome

Translation mixtures with MuLV RNA were fortified with purified yeast suppressor tRNA at 40  $\mu$ g/ml. (A) No addition; (B) yeast nonsuppressor tRNA; (C) yeast ochre suppressor tRNA; (D) yeast amber suppressor tRNA. The suppressor activity of the yeast tRNA was controlled by concurrent translation of a Q $\beta$  (Q $\beta$  aml) mutant in the same cell-free system. (E) No addition; (F) ochre suppressor tRNA; (G) amber suppressor tRNA. The molecular weights of the polypeptides synthesized are indicated as in Figure 2.

by the amber tRNA. Yeast ochre suppressor tRNA increased the Pr180<sup>gag-pol</sup> somewhat and also depressed the intensity of Pr78<sup>gag</sup> (Figure 5, slot B).

To establish whether the 180K product stimulated by amber tRNA was truly Pr180<sup>gag-pol</sup>, immunoprecipitation studies of the suppressed products were performed. From the original translation mixture (Figure 6, slot E), anti-p30 precipitated the 180K band and others of nearly that size, as well as the 65K protein and some residual 78K protein (Figure 6, slot A). Very little 110K protein was precipitated. Purified p30 competed all of this precipitation (Figure 6, slot B). Anti-reverse transcriptase, in the presence of purified p30, precipitated only the 110–180 K polypeptides (Figure 6, slot C), and the purified enzyme competed this precipitation (Figure 6, slot D). Thus the 180K polypeptide made in the presence of amber suppressor tRNA appears to be Pr180<sup>gag-pol</sup>, and the 110K may be Pr110<sup>pol</sup>.

Translation in the nuclease-treated reticulocyte system of Q $\beta$  RNA with an amber mutation in the synthetase gene gave no detectable complete synthetase (Figure 5, slot E). Addition of yeast ochre suppressor tRNA stimulated some synthesis of the complete product (Figure 5, slot F) while the amber suppressor tRNA produced a 3 fold higher yield of the synthetase protein (Figure 5, slot G). The MuLV gag termination signal, therefore, behaved identically to an established UAG codon in its response to the yeast suppressor tRNAs. Gesteland et al. (1977) had previously shown that a mutant codon in an adeno-SV40 hybrid behaved in this fashion and concluded that it was a UAG. We therefore believe it very probable that the gag/pol junction of MuLV RNA contains a UAG codon.

#### Effect of Suppressor tRNA on Translation of MSV RNA

MSV RNA appears to be a deleted MuLV RNA genome with insertion of mouse cellular sequences (Frankel et al., 1976; Hu et al., 1977). Because of the possibility that MSV RNA might also contain a suppressible codon, we studied its translation. In the nuclease-treated reticulocyte system, MSV RNA directed synthesis of a major 62K band and lesser amounts of bands of 72K, 42K, 40K, 32K and 18K daltons (Figure 2, slot C). The 62K band has been seen in MSV-transformed cells (Robey et al., 1977) and in virions of MSV(FeLV) (Oskarsson et al., 1975), and has the antigenicity of the MuLV gag proteins.

Immunoprecipitation with anti-p30 of the products formed under the direction of MSV RNA showed that the 72K, 62K, 55K and 40K polypeptides had p30 antigenicity (Figure 7, slot A). Purified p30 competed the vast majority of this precipitation (Figure 7, slot B). In the presence of p30,

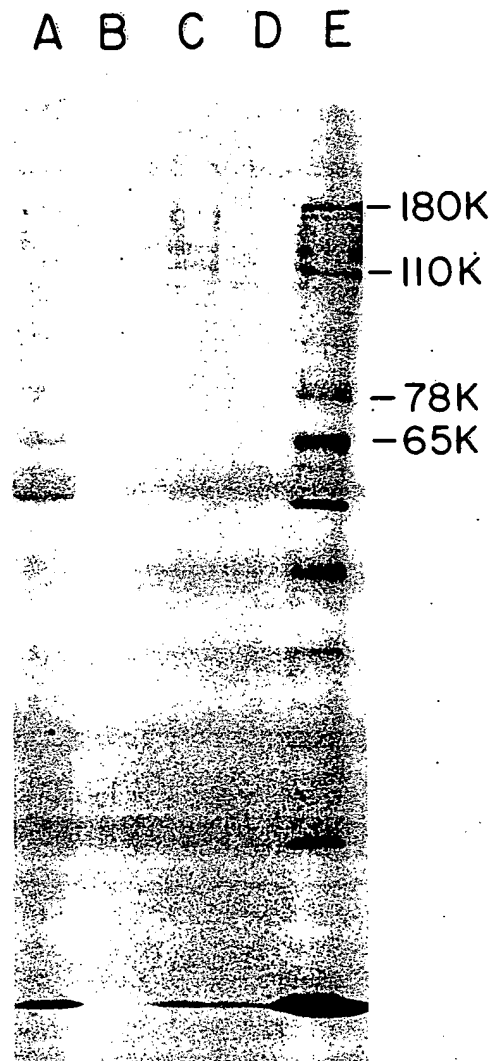


Figure 6. Immunoprecipitation of Polypeptides Generated from MuLV RNA in the Presence of Yeast Amber Suppressor tRNA

A translation mixture with MuLV RNA containing yeast amber suppressor tRNA was immunoprecipitated with antisera against MuLV proteins. (A) Immunoprecipitation with anti-p30 serum; (B) immunoprecipitation with anti-p30 serum in the presence of an excess of purified p30; (C) immunoprecipitation with anti-reverse transcriptase serum in the presence of an excess of purified p30; (D) immunoprecipitation with anti-reverse transcriptase antiserum in the presence of an excess of purified p30 and reverse transcriptase; (E) original translation mixture.

neither anti-reverse transcriptase (Figure 7, slot C) nor anti-glycoprotein (Figure 7, slot D) gave detectable specific bands.

Two of the polypeptides generated in significant amounts by MSV RNA were not encoded by MuLV RNA and were not immunoprecipitable with the available antisera—42K and 18K (Figure 2C). Harvey sarcoma virus can also specify a polypeptide, in this case 24K, which is not found in the products encoded by the corresponding leukemia

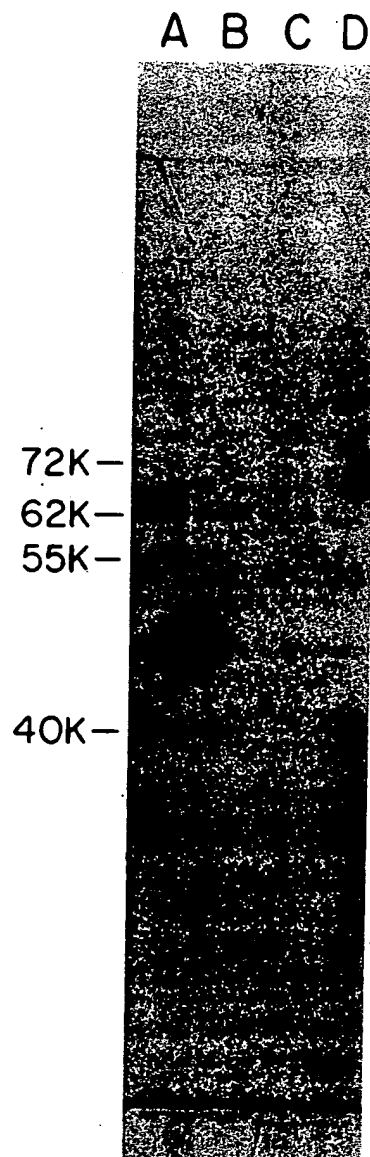


Figure 7. Immunoprecipitation of Polypeptides Generated by MSV RNA in the Cell-Free System

A translation mixture with MSV RNA (Figure 2C) was immunoprecipitated with antibodies against the structural proteins of MuLV. (A) Immunoprecipitate with anti-p30 serum; (B) immunoprecipitation with anti-p30 serum in the presence of purified p30; (C) immunoprecipitation with anti-reverse transcriptase serum in the presence of purified p30; (D) immunoprecipitation with anti-glycoprotein serum in the presence of purified p30. The molecular weights of the polypeptides are indicated as in Figure 2.

virus (Parks and Scolnick, 1977), and it is not immunoprecipitable with our reagents (U. Olshevsky, R. Weinberg and D. Baltimore, unpublished results).

When an MSV RNA-programmed extract was supplemented with yeast amber tRNA, the minor 72K in the control product (Figure 8, slot A) was

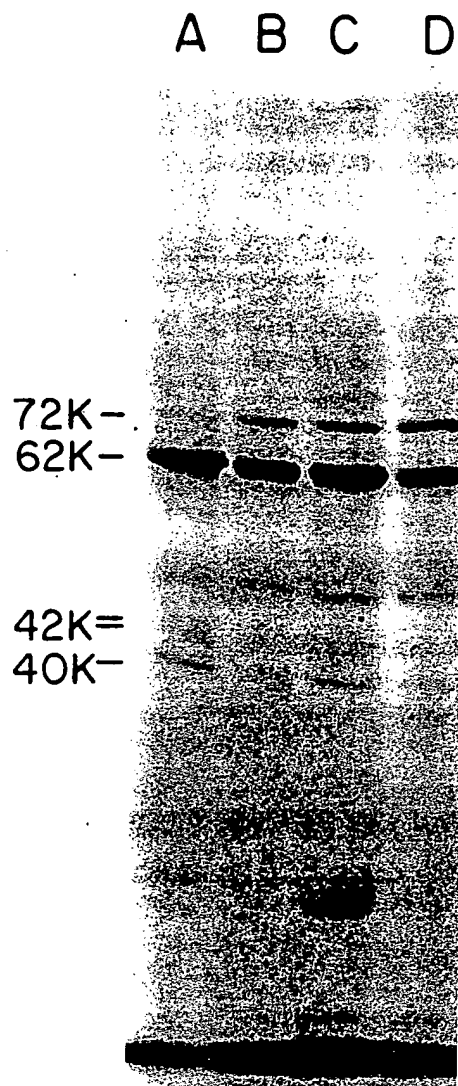


Figure 8. The Effect of Yeast Suppressor tRNA on the Translation of MSV RNA in the Cell-Free System

Translation mixtures with MSV RNA were fortified with different yeast suppressor tRNAs at 40  $\mu$ g/ml. (A) No addition; (B) amber suppressor tRNA; (C) ochre suppressor tRNA; (D) amber and ochre suppressor tRNA.

greatly accentuated (Figure 8, slot B) and the major 62K product was diminished somewhat. Ochre tRNA also increased the 72K, but without any evident diminution of the 62K (Figure 8, slot C), and a mixture of the two tRNAs gave the largest amount of the 72K product (Figure 8, slot D). Immunoprecipitation of the products made in the presence of amber tRNA showed that 72K and 62K both had p30 determinants, but that no products with reverse transcriptase or glycoprotein determinants were evident (Figure 9).

It therefore appears that a UAG codon terminates the 62K product synthesized under the direction of

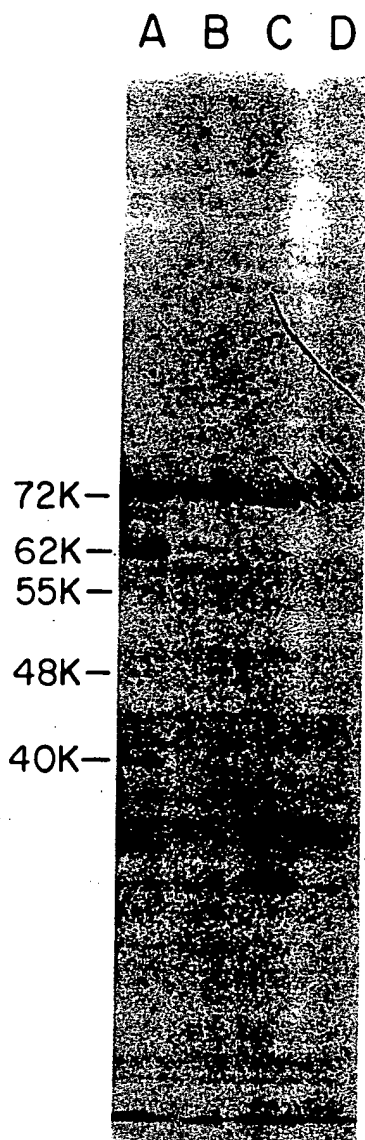


Figure 9. Immunoprecipitation of Polypeptides Generated from MSV RNA in the Presence of Yeast Amber Suppressor tRNA

A translation mixture with MSV RNA containing yeast amber suppressor tRNA was immunoprecipitated with antisera against MuLV proteins. (A) Immunoprecipitation with anti-p30 serum; (B) immunoprecipitation with anti-p30 serum in the presence of purified p30; (C) immunoprecipitation with anti-reverse transcriptase serum in the presence of purified p30; (D) immunoprecipitation with anti-glycoprotein serum in the presence of purified p30.

MSV RNA, and that its suppression generates a 72K product.

## Discussion

### The Cell-Free Products

The polypeptides specified by MuLV 35S RNA in the nuclease-treated reticulocyte lysate system include the major ones previously seen in systems

derived from tissue culture cells (Naso et al., 1975; Kerr et al., 1976). The advantage of the nuclease-treated extracts is their low background which has allowed smaller and quite minor products to be recognized. Nuclease-treated tissue culture cell extracts give patterns similar to those generated by treated reticulocyte extracts (U. Olshevsky, unpublished results).

Immunoprecipitation has identified four classes of MuLV-specific polypeptides made in the cell-free system:

- Products with only *gag* protein antigenicity (reactivity with anti-p30 serum) — Pr78, Pr65, 52K and 40K. The larger two have in vivo counterparts (Jamjoom et al., 1977; our unpublished data), and the smaller may result from premature termination or be cleavage products of the larger ones. Why there are two major *gag* products is not clear; from kinetic experiments in the cell-free system, Pr65 does not appear to be derived from Pr78 (unpublished results), but Pr65 does appear to result from cleavage of Pr78 in infected cells (Arcement et al., 1976, 1977).
- A product with both *gag* and *pol* antigenicity — Pr180. This has an in vivo counterpart which is probably the major precursor of reverse transcriptase (Jamjoom et al., 1977; Oppermann et al., 1977). There is also a series of smaller variable minor bands with molecular weights higher than 110,000 daltons that have both *gag* and *pol* reactivity.
- A product in which we have detected only *pol* reactivity — Pr110. This protein appears to be about the size of the smaller reverse transcriptase precursor found in infected cells (Jamjoom et al., 1977), although its identity with the in vivo *pol* precursor remains to be demonstrated.
- A minor product with only *env* antigenicity — P70. This is presumably the unglycosylated form of Pr90, the precursor of both gp70 and P15(E), the envelope proteins. It may result from a slight contamination of the 35S RNA with a smaller species packaged in virions, or it may be translated from broken 35S RNA molecules; 35S RNA apparently cannot generate envelope protein (Van Zaane et al., 1977).

The cell-free system mimics the in vivo situation quantitatively as well as qualitatively. There is about 50 times as much of the Pr65<sup>gag</sup> + Pr78<sup>gag</sup> made as there is of Pr180<sup>gag-pol</sup>, conforming to the estimates of this ratio in infected cells (Panet et al., 1975; Jamjoom et al., 1977). Thus the in vitro system poses the same question of *gag/pol* junction control raised by the in vivo data discussed in the Introduction.

### Suppression

The ability of amber suppressor tRNA<sup>ser</sup> to increase synthesis of Pr180<sup>gag-pol</sup> apparently at the expense

of Pr78<sup>sup</sup> suggests that translation of Pr78<sup>sup</sup> is terminated by a UAG codon. Such an hypothesis is consistent with the ability of the purified ochre (UAA) suppressor to give a lower level of read-through because when it is purified, ochre suppressor tRNA<sup>ser</sup> is known to read a UAG codon with significant efficiency (Gesteland et al., 1977). By contrast, crude tRNA from an ochre suppressor strain does not suppress a UAG codon (Gesteland et al., 1977), and more recent preparations of purified ochre suppressor tRNA<sup>ser</sup> neither suppressed the Q $\beta$  amber mutation nor gave suppression in the MuLV system (unpublished results). A yeast suppressor tRNA tentatively identified as a suppressor of termination of UGA had no effect on MuLV RNA translation (unpublished results).

In the cell-free system, both Pr65<sup>sup</sup> and Pr78<sup>sup</sup> are formed, but only Pr78<sup>sup</sup> is decreased when suppressor tRNA is added. It appears that Pr65<sup>sup</sup> arises by termination before completion of Pr78<sup>sup</sup> so that its synthesis stops before reaching the suppressible codon. The Pr65<sup>sup</sup> found in infected cells is believed to be cleaved from Pr78<sup>sup</sup> (Arcecent et al., 1976, 1977), and thus the exact relationship of the in vivo and in vitro products requires clarification.

The ability of added yeast suppressor tRNA to cause a read-through from the *gag* to the *pol* region does not necessarily explain how the normal level of a small percentage of read-through is achieved. The normal read-through could, for instance, be suppression resulting from a ribosome-mediated event or from the action of a soluble protein. Ribosome- and drug-mediated suppression has been described for bacterial systems (Gorini, 1970). The simplest hypothesis is, however, that cells have a low normal level of suppressor tRNA. This need not be induced by virus infection because the cell-free system in the present studies, which was derived from normal rabbit reticulocytes, allowed some read-through. If normal suppressor tRNA exists, it should be possible to identify it using MuLV RNA translation as an assay. Cellular as well as viral proteins could be made by read-through of suppressible codons.

We are unable to interpret easily the suppression of MSV RNA translation which allows the apparent formation of a 72K product from an original 62K product. The MSV genome contains much of the *gag* region of the parental MuLV genome. If the UAG codon that terminates the 62K protein is the same one that terminates the 78K MuLV product, a small deletion inside the *gag* region would have to be present. Because suppression in the MuLV system generates a 180,000 dalton product, there would then have to be another mutation or deletion beyond the UAG in MSV RNA to account for termination of the suppressed product at 72K. Alterna-

tive models are easily conceived, and the existing heteroduplex analyses (Hu et al., 1977) do not provide the resolution to choose among them.

### MSV Products

The MSV RNA translation products include two that are not immunoprecipitable with the tested sera. These sera precipitated all the MuLV RNA-directed products. Translation of Harvey sarcoma virus RNA had previously been shown to generate a 24K product (Parks and Scolnick, 1977); it was not immunoprecipitable with our sera and appears to lack peptides in common with leukemia virus proteins (U. Olshevsky, R. Weinberg and D. Baltimore, unpublished results). The role of these sarcoma virus-specific polypeptides in the sarcomagenic properties of the viruses must await further studies on the origin of these polypeptides.

### Experimental Procedures

#### Cell, Virus and Viral RNA

The MuLV RNA used in these studies was derived from clone 2 Moloney MuLV which was obtained from clone 1 virus (Fan and Paskind, 1974) by end-point dilution of the virus on NIH/3T3 with immediate cloning of the cells after infection (M. Paskind, D. Smotkin and D. Baltimore, unpublished data). Moloney sarcoma virus (MSV) was harvested from a murine cell line, TB(CFW/D) clone 124, which overproduces sarcoma virus against a low background of the Moloney MuLV helper (Ball et al., 1973; Maisel, Dina and Duesberg, 1977). Large-scale production of rapid harvest virus was achieved by growing both cell lines in roller bottles in Dulbecco's modified Eagle's medium (DME) with 10% calf serum. Virus was in most cases purified from medium collected continuously or at 4 hr intervals. The medium was clarified by centrifugation at 1000  $\times$  g for 15 min at 4°C, and the virions were collected by centrifugation at 27,000  $\times$  g for 2.5 hr. The pellets were resuspended in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, and centrifuged to density equilibrium in 25-40% (w/w) sucrose gradients for 17 hr at 82,000  $\times$  g at 4°C. Banded virus was pooled and diluted 3 fold in 7 M urea, 0.35 M NaCl, 2% SDS, 10 mM EDTA and 0.1 M Tris-HCl (pH 7.8). The suspension was extracted with a 1:1 mixture of phenol and chloroform as described by Holmes and Bonner (1973), and the RNA was precipitated with ethanol. The pellets were redissolved, and both 70S and 35S MuLV RNA as well as 50S and 30S MSV RNA were isolated by two consecutive sucrose gradients as previously described (Kerr et al., 1976). The preparation of RNA from the Q $\beta$  wild-type phage and the amber mutant (Q $\beta$  am1) has been described (Gesteland et al., 1976).

#### Cell-Free Protein Synthesis

Reticulocyte extracts treated with micrococcal nuclease to reduce endogenous mRNA were used throughout (Pelham and Jackson, 1976). Dr. Bryan Roberts (Massachusetts Institute of Technology) supplied most of the extract used in this study. Protein synthesis was carried out in a total volume of 25  $\mu$ l containing 10  $\mu$ l of reticulocyte extract fortified with 0.2 mM hemin, 0.16  $\mu$ g of creatine phosphokinase, 80 mM potassium acetate, 1.25 mM Mg-acetate, 8 mM creatine phosphate, 0.5 mM spermidine, 2 mM DTT, 20 mM Hepes buffer (pH 7.6), 20  $\mu$ M of all amino acids except methionine and 25  $\mu$ Cl of <sup>35</sup>S-methionine (Amersham, England). Rabbit tRNA (Gibco) was in most cases added to a final concentration of 40  $\mu$ g/ml, and yeast suppressor tRNAs were also included at final concentrations of 40  $\mu$ g/ml. Incubation was carried out at 35°C for 1-2 hr, and synthesis was linear for at least 1 hr. At the end of the incubation period, the reaction

mixture was diluted 2 fold in 1% Triton X-100, 0.5% Na-deoxycholate (DOC), 0.1% SDS, 10 mM phosphate buffer (pH 7.4), and frozen at  $-20^{\circ}\text{C}$  awaiting analyses by SDS-polyacrylamide electrophoresis or immunoprecipitation.

#### Antisera against MuLV Proteins

Rabbit antisera were produced against three of the major proteins of MuLV virions. Proteins were purified from 200 mg of clone 1 MuLV (Fan and Paskind, 1974) following isopycnic banding in sucrose gradients. Virions at 5 mg/ml were lysed in 100 mM Tris-HCl (pH 8), 200 mM KCl, 0.1 mM EDTA, 20 mM dithiothreitol and 1% NP-40. Following clarification at 10,000 rpm for 30 min, the supernatant was dialyzed against buffer A [50 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 20% glycerol, 0.1% NP-40] plus 0.1 M KCl. The preparation was freed of nucleic acid by passage through DEAE-Sephadex A-25. Protein gp70 was then purified by passage through phosphocellulose in buffer A following by binding to concanavalin A-Sepharose in phosphate-buffered saline and elution with 0.3 M  $\alpha$ -methylmannoside. The protein was finally fractionated by preparative SDS-polyacrylamide gel electrophoresis (Lazarides and Hubbard, 1976). The major core protein (p30) was purified by gradient elution from phosphocellulose followed by preparative SDS-polyacrylamide gel electrophoresis. The reverse transcriptase was purified by gradient elution from phosphocellulose followed by gradient elution from double-stranded DNA cellulose and concentration by binding and step elution from phosphocellulose (Haseltine et al., 1977). From 100–150  $\mu\text{g}$  of the purified polypeptides were mixed with complete Freund's adjuvant and injected into rabbits. After one booster injection 15 days later of a similar amount of antigen in incomplete adjuvant, the animals were bled over the following 2 weeks. Because the anti-reverse transcriptase and the anti-gp70 sera also contained small amounts of antibodies to p30 (not evident in Figure 3), an excess of purified unlabeled p30 was added to immunoprecipitation with these antisera.

#### Immunoprecipitation

Samples (10  $\mu\text{l}$ ) from the cell-free translation mixture were diluted 5–10 fold into 0.1 M NaCl, 10 mM phosphate buffer (pH 7.4), 1% Triton X-100, 0.5% DOC, 0.1% SDS and 1–2  $\mu\text{l}$  of the specific antiserum. The mixture was incubated for 1 hr at room temperature and overnight at  $4^{\circ}\text{C}$ . A 10% (v/v) preparation of formaldehyde-treated *Staphylococcus aureus* (strain Cowan-1) was prepared in the dilution buffer with 1 mg/ml of bovine serum to prevent unspecific binding of immune complexes to the bacterial cell wall. From 10–20  $\mu\text{l}$  of bacterial suspension were added, and the incubation continued for 2–4 hr at  $4^{\circ}\text{C}$ . The bacteria were subsequently washed 2 or 3 times in the dilution buffer by centrifugation, resuspended in the sample buffer and boiled for 3–4 min; the supernatant was analyzed by gel electrophoresis. A detailed description of the method of indirect immunoprecipitation with bound protein A of *Staphylococcus aureus* has been published (Kessler, 1975).

#### Agarose Gel Electrophoresis of RNA

Isolated viral RNA was analyzed analytically on 1% agarose slab gels at 5 V/cm for 2 hr in 10 mM Tris-acetate (pH 7.2), 1 mM EDTA as described by Sharp, Sugden and Sambrook (1973). The procedure of Bailey and Davidson (1976) was followed for methylmercuric hydroxide gels, except that 1% agarose slab gels were used. Methylmercuric hydroxide was obtained from Alfa Products (Danver, Massachusetts) as a 1 M liquid stock solution.

#### SDS-Polyacrylamide Gel Electrophoresis

Samples from the translation mixtures were diluted 2 fold to reduce the salt concentration and mixed with equal volumes of a twice-concentrated sample buffer yielding a final concentration of 2% SDS, 2% 2-mercaptoethanol, 60  $\mu\text{M}$  Tris-HCl (pH 6.8) and 20% glycerol. After boiling for 3–4 min, the samples were analyzed by electrophoresis on 10% polyacrylamide slab gels containing

0.1% SDS (Laemmli, 1970). Autoradiography of the dried polyacrylamide gels was with Kodak Xomat film XR5. To quantitate the relative amounts of radioactivity in the different bands, the films were scanned by a densitometer and the peak area was determined for each peak. The same amount of the translation mixture was applied to each gel slot.

#### Suppressor tRNA from Yeast

The yeast strains DBA317 and H159 originally isolated by Brandriss, Soll and Botstein (1975) and Hawthorne and Leupold (1974) were used to isolate enriched amber and ochre tRNA, respectively. Serine tRNA was also isolated from DBD195, the nonsuppressor parent strain of DBA317. Total RNA was extracted, and uncharged tRNA was isolated as previously described (Gesteland et al., 1976). The tRNA from all strains was enriched for the serine tRNA by sequential chromatography on BD-cellulose and Sepharose 4B (Gesteland et al., 1976). The suppressing activity, like the major serine tRNA species, was retarded during both chromatography steps. The final pooled fractions were about 50% pure with respect to their ability to accept serine, but the suppressor tRNA represents an unknown, although probably small, fraction of the total tRNA present. In some experiments, an apparent UGA suppressor tRNA from a yeast strain (5307) was also used. Purified tRNA was added at 20–40  $\mu\text{g}/\text{ml}$  to the translation mixture.

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# Cell





## Visualization of Single-Stranded Nucleic Acid of RNA Tumor Virus With The Electron Microscope

(avian myeloblastosis virus/denaturation)

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**ABSTRACT** 60-70S RNA of avian myeloblastosis virus was spread with the Kleinschmidt method and examined in the electron microscope after being subjected to a variety of denaturing conditions. Extension of the molecules from a collapsed configuration was seen only under stringent denaturing conditions, resulting in molecules of 1-2  $\mu$ m in length. Longer strands of these extremely fragile molecules cannot be obtained with the methods presently available.

It has been postulated that the high-molecular-weight RNA of oncornaviruses may be composed of 3 or 4 subunit molecules (1-6), linked by small hydrogen-bonded, double-stranded regions (7). Smaller RNA molecules have also been found to be associated with the 60-70S high-molecular-weight RNA (8, 9). Publications on the ultrastructure of the RNA from a variety of oncornaviruses are few, understandably so in light of the complex nature of the molecule. Granboulan, Huppert, and Lacour (10), using the Kleinschmidt spreading technique (11), first showed the structure of RNA from avian myeloblastosis virus (AMV) with molecules of up to 10  $\mu$ m in length. Similar results were obtained by Kakefuda and Bader (12) in examining the RNA from murine leukemia virus. Recently Whalley (13) described molecules from feline leukemia virus as being comparable to those observed by Granboulan. However, Sarkar and Moore (14) found only relatively short molecules associated with mouse mammary tumor virus. But these, it was suggested, may represent the subunit moieties.

Problems of spreading RNA molecules from oncornaviruses are numerous. Our initial attempts, using the high-molecular-weight RNA from plasma AMV, together with published techniques of spreading (10-12, 14-16, 18), were nonproductive: long, extended strands identical to those previously reported for other oncornavirus RNA (10, 12-14) were found to be contaminating duplex DNA that was always present in small amounts in the 60-70S high-molecular-weight RNA fractions (manuscript in preparation). However, recently published techniques now allow for a direct visual discrimination of single- compared to double-stranded molecules (18). Because of random base interactions, single-stranded molecules must be subjected to some form of denaturation before extended strands can be seen. Duplex molecules apparently need no denaturation. Unfortunately, most authors have reported long strands of oncornavirus "RNA" under conditions suitable only for the extension of

duplex molecules (10, 12-14). Thus the evidence indicates that previously published illustrations represent the contaminating strands of duplex DNA.

The following report presents experiments demonstrating the ultrastructure of 60-70S RNA from AMV under a variety of spreading conditions.

### MATERIALS AND METHODS

AMV RNA was isolated from plasma virus, supplied by Dr. J. W. Beard (Life Sciences, Inc., St. Petersburg, Fla.), by a sodium dodecyl sulfate-phenol extraction. Virus was first concentrated from the plasma by isopycnic centrifugation in a sucrose gradient in TNE buffer (10 mM Tris, pH 8.0-0.15 M NaCl-1 mM EDTA). The visible virus band was collected, diluted with TNE buffer, pelleted by centrifugation, and resuspended in buffer. Sodium dodecyl sulfate was added to give 1% (w/v). This was followed by the addition of an equal volume of buffer-saturated phenol containing 0.1% (w/v) 8-hydroxyquinoline. The mixture was kept at 4° for 10 min with periodic agitation. Aqueous and phenol phases were separated by centrifugation at 18,000  $\times g$  for 10 min. The phenol phase was removed and the extraction repeated. The final aqueous phase was twice extracted with cold anhydrous ether, and samples were then taken for Kleinschmidt spreading and analysis by sedimentation velocity centrifugation. In some preparations extraction of the RNA with ether was replaced with dialysis against TNE buffer.

Grids of AMV RNA were prepared by modifications of the protein monolayer techniques of Kleinschmidt and Zahn (15), Westmoreland *et al.* (16), and Robberson *et al.* (17). Four different spreading conditions were used, each varying in its capacity to denature the RNA molecules. (I) RNA to be spread was initially diluted 1:10 with 4 M urea. To 25  $\mu$ l of the diluted RNA, the following were added: 20  $\mu$ l of 1 M ammonium acetate and 5  $\mu$ l of cytochrome *c* (1 mg/ml); the hypophase was 15 mM ammonium acetate. (II) As described in I, with the exception that the RNA was originally diluted with 8 M urea. (III) Five microliters of RNA were diluted with 40  $\mu$ l of 0.8 M urea prepared in 99% formamide (Matheson, Coleman, and Bell, East Rutherford, N.J.). Then 5  $\mu$ l of cytochrome *c* (0.5 mg/ml in 0.5 M Tris, pH 8.0-50 mM EDTA) were added. The hypophase consisted of: 10 mM Tris, pH 8.0-1 mM EDTA. (IV) As described in III, with the exception that the RNA was diluted with 4 M urea made in 99% formamide. The concentration of RNA in all spreading solutions was approximately 0.33

Abbreviation: AMV, avian myeloblastosis virus.

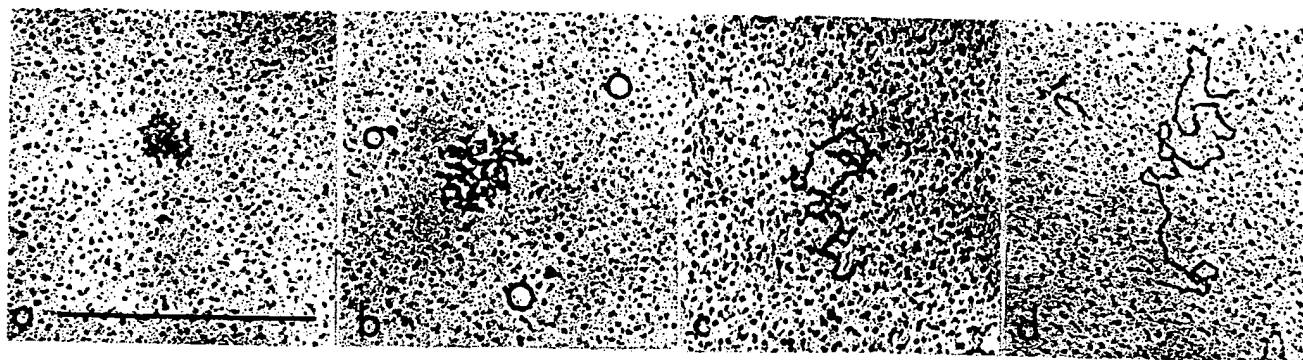


FIG. 1. Electron micrographs of 60-70S AMV RNA. AMV RNA was fractionated in a 10-30% (v/v) glycerol gradient made in TNE buffer. Centrifugation was for 80 min at 50,000 rpm in an SW50 rotor. Fractions were collected and samples were taken for both  $A_{260}$  determinations and spreading. (a) RNA initially diluted with 4 M urea, then spread in the presence of 0.4 M ammonium acetate and cytochrome c. (b) As described in a, with the exception that the RNA was diluted with 8 M urea. (c) RNA spread in the presence of approximately 0.8 M urea and 80% formamide. (d) RNA spread in 4 M urea and 80% formamide. The bar represents 1.0  $\mu$ m.

$\mu$ g/ml. All solutions to be spread were applied to an appropriate hypophase by a glass slide, according to the aqueous technique described by Davis *et al.* (18). The RNA protein film was picked up onto the surface of Parlodion-covered grids, stained with uranyl acetate (18), and rotary shadowed with platinum-palladium at an angle of approximately  $8^\circ$ . The grids were examined as described (19).

#### RESULTS AND DISCUSSION

Examples of our results with 60-70S AMV RNA spread under the above conditions are presented in Fig. 1. Dilution of the RNA with only 4 M (Fig. 1a) or 8 M urea (Fig. 1b) had no noticeable effect on the configuration of the collapsed molecule. Extension of the molecules from the collapsed configuration occurred only under the combined denaturing effects of both urea and formamide (Fig. 1c). Fully extended

molecules were observed under the conditions of Robberson *et al.* (17), i.e., approximately 4 M urea and 80% formamide (Fig. 1d).

An examination of molecule length at various stages of "unraveling" resulted in the longest molecules being observed under the most stringent of denaturing conditions (Figs. 1d, 2, and 3). A histogram of extended 60-70S AMV RNA

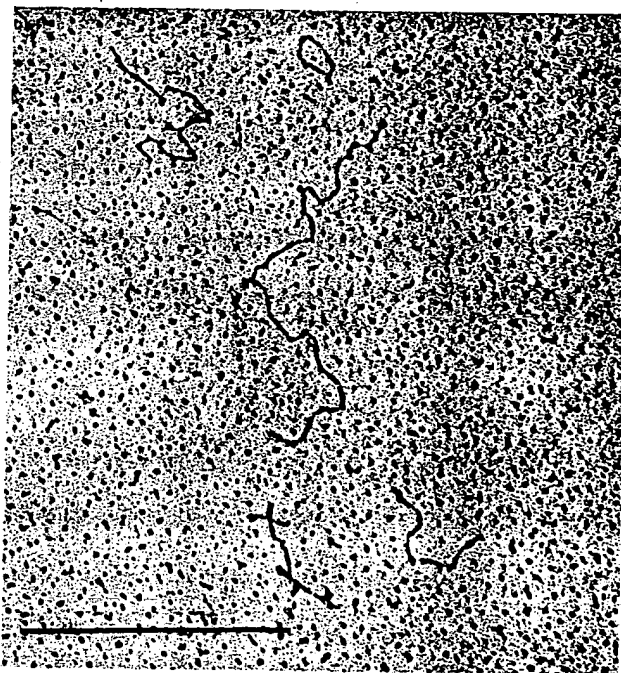


FIG. 2. AMV 60-70S RNA spread in the presence of 4 M urea and 80% formamide. The bar represents 1.0  $\mu$ m.

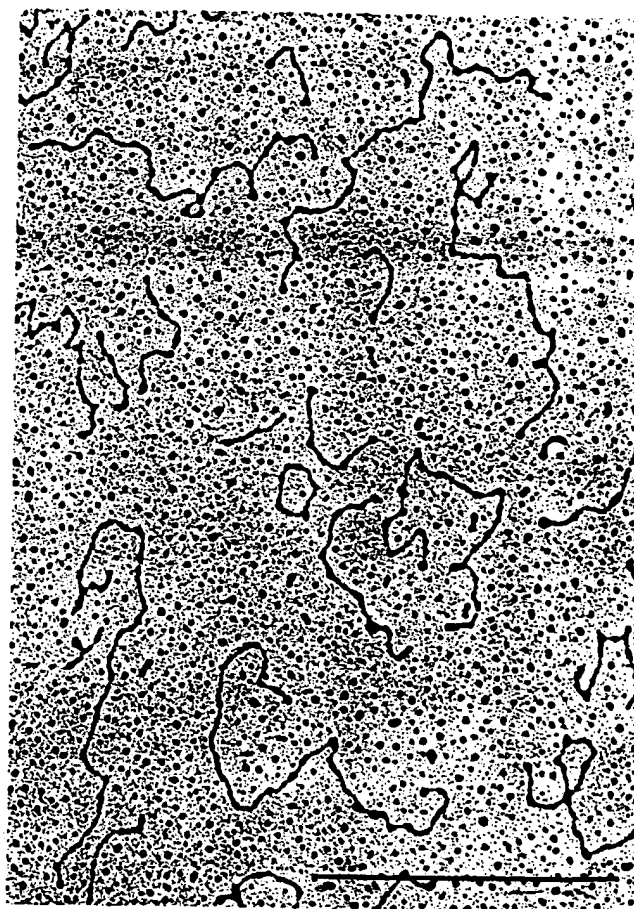


FIG. 3. Unfractionated AMV RNA spread as described in Fig. 2. The bar represents 1.0  $\mu$ m.

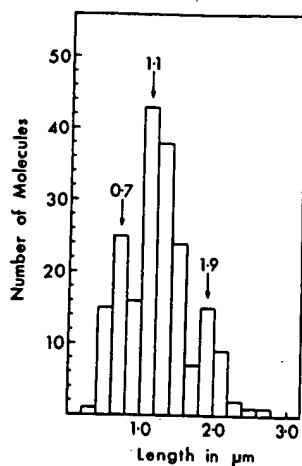


Fig. 4. Histogram of AMV 60-70S RNA spread as described in Fig. 2. A total of 197 molecules was measured.

molecules is presented in Fig. 4, showing modal lengths of 0.7  $\mu\text{m}$ , 1.1  $\mu\text{m}$ , and 1.9  $\mu\text{m}$ . A total of 197 molecules was measured. Molecular weight determinations (20) of the molecules observed here resulted in values of  $0.7 \times 10^6$ ,  $1.2 \times 10^6$ , and  $2.2 \times 10^6$ .

On the assumption that the conditions used here for extending the molecules may also have led to a disruption of covalent bonds, isolation and purification techniques identical to those used for AMV RNA were used to examine the nonsegmented RNA from vesicular stomatitis virus (molecular weight  $3.2 \times 10^6$ ) (21) and Newcastle disease virus (molecular weight  $5.2$  to  $5.6 \times 10^6$ ) (22). Spread under the above conditions (IV), both RNAs exhibited lengths that correspond closely to their molecular weights (manuscript in preparation) (Fig. 5).

It is apparent, then, that AMV 60-70S RNA is not a linear covalently-bonded molecule of 8-10  $\mu\text{m}$ . However, molecules of the length seen here may represent subunits previously linked by short stretches of hydrogen bonding (7). The extensive denaturation used to "stretch" the molecule may have also disrupted these double-stranded regions. To date, the only visual evidence of possible double-strandedness within the molecule occurs with the appearance of loops or folded regions similar to those demonstrated by Wellauer and Dawid (23) for ribosomal RNA.

An alternative explanation of the results seen here is that the RNA sedimenting at 60-70S may consist of a complex of discrete, short molecules in an extremely collapsed configuration. Denaturation of 60-70S RNA would change, as is seen here, its tertiary structure, which would result in changes in its sedimentation constant (1-4) and electrophoretic mobility (1, 3, 5). The irreversibility of the denaturation process (4) seems to substantiate this arrangement of molecules in the virion.

The question of the true length of the intact viral genome may still be unanswered. Methods used to extend the molecule for length determination may irreversibly disrupt the genome. An ultrastructural analysis of the high-molecular-weight RNA in the absence of denaturing agents should be done, i.e., by examining the duplex RNA-DNA hybrid formed by the RNA-directed DNA polymerase of RNA tumor virus (24).

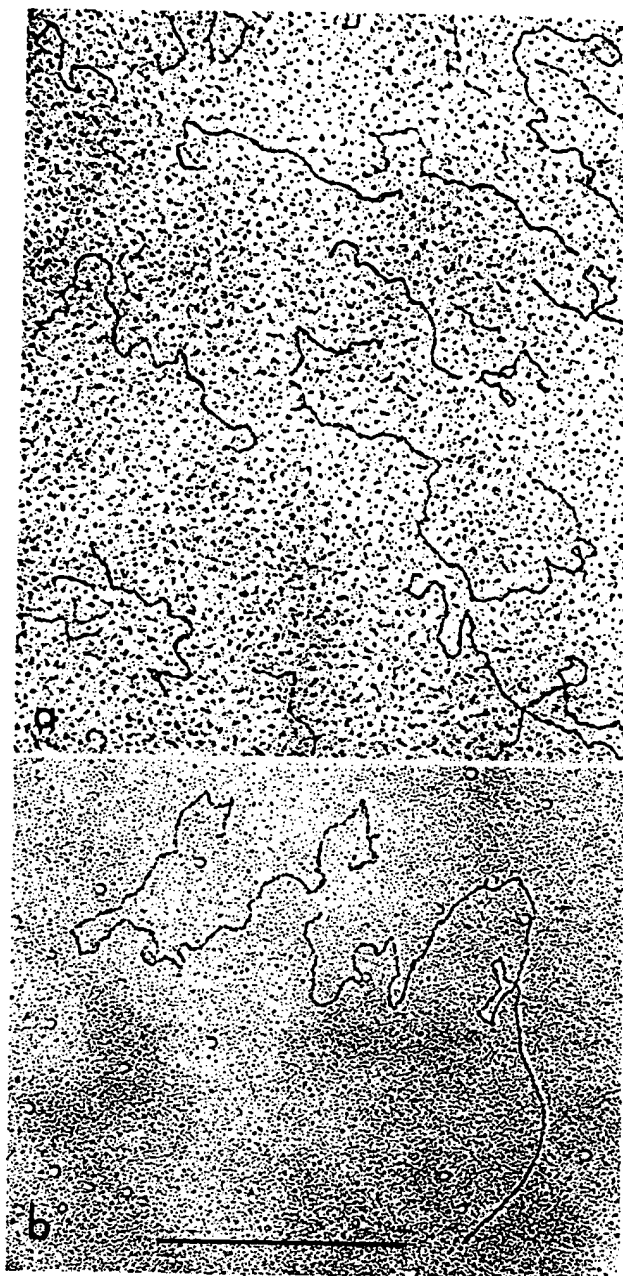


Fig. 5. Electron micrographs of the RNA from vesicular stomatitis virus (a) and Newcastle disease virus (b) spread in the presence of urea and formamide. Measured length corresponds to a molecular weight of approximately  $3.2 \times 10^6$  for RNA of vesicular stomatitis virus and  $5.0 \times 10^6$  for RNA of Newcastle disease virus. The bar represents 1.0  $\mu\text{m}$ .

We thank Messrs. D. Jones and B. Elliott, Jr. for their skillful technical help and Dr. C. Garon for valuable discussions.

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# Mapping of Poly(A) Sequences in the Electron Microscope Reveals Unusual Structure of Type C Oncornavirus RNA Molecules

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## Summary

We have synthesized a convenient electron microscope label for mapping poly(A) sequences. Short lengths of poly(dT) are polymerized onto nicked circular SV40 DNA with the enzyme terminal deoxynucleotidyl transferase. An RNA or DNA molecule of interest is treated with glyoxal, hybridized briefly with the poly(dT) circles, and spread for microscopy; poly(A) stretches are clearly marked because they are attached to the poly(dT) on the easily recognized SV40 duplex circles.

The RNAs of several type C oncornaviruses were examined by this method. The endogenous feline virus (RD-114), the endogenous baboon virus (BKD), and the woolly monkey sarcoma virus (WoMV) all contain a dimer of RNA subunits held together in a central secondary structure feature we call the dimer linkage structure. Both ends distal to the dimer linkage structure hybridize to the SV40-poly(dT). Assuming both poly(A)s are on the 3' ends of the subunits and that both subunits are identical, the two identical subunits are held together by interactions between sequences close to the 5' ends.

## Introduction

We have developed a reliable, efficient method for identifying and mapping poly(A) stretches in nucleic acids by electron microscopy. We have used this technique to study the structure of the RNA of RD-114 and other tumor viruses.

Electron microscope studies in this laboratory (Kung et al., 1975; Kung et al., 1976) show that the high molecular weight RNA molecules extracted from several different RNA tumor viruses are dimers, with reproducible secondary structures. Each of the two monomer subunits of any one dimer has a molecular length of approximately 10 kb (kb = kilobase, 1000 nucleotides).

In the dimer, an end of one monomer is noncovalently joined to an end of the other monomer in a central secondary structure feature. This feature in RD-114 RNA was previously called the "rabbit ears" (Kung et al., 1975), but because its shape is different in different tumor virus RNAs, we now prefer the more general term, dimer linkage structure. Each dimer thus has two monomer ends joined in the dimer linkage structure and two free ends. We

have determined the positions of poly(A) sequences in the dimers and in dissociated monomers.

The obvious method for electron microscope mapping of poly(A) stretches in a nucleic acid strand is to hybridize the poly(A) to a poly(dT) or poly(U) strand attached to a suitable electron microscope label. The initial problem encountered is that A·T and A·U base pairs are relatively unstable, so that the moderately denaturing conditions needed to extend single-strand nucleic acids in electron microscope spreads cause dissociation of the poly(A)·poly(dT) or poly(A)·poly(U) duplexes. This difficulty was overcome by Hsu, Kung, and Davidson (1973) by treating the nucleic acid with glyoxal. Under suitable reaction conditions, glyoxal is bound only to G residues and blocks their base pairing capability (Broude and Budowsky, 1971). This reaction disrupts the secondary structure of most nucleic acids sufficiently so that they can be extended for electron microscopy by weakly denaturing solvents (such as 30% formamide, 0.1 M Tris) in which poly(A)·poly(dT) duplexes are stable. Hsu and his co-workers used long poly(dT) strands to label the poly(A) sequences at the end of Sindbis virus RNA. This method was moderately effective, but discrimination between the poly(dT) strand and the Sindbis RNA strand was not always certain. Lizardi, Williamson, and Brown (1975) hybridized poly(dT) to glyoxal-treated silk fibroin mRNA, then added excess poly(A), and observed the resulting duplex regions at the ends of some of the mRNA molecules. In this method, the duplex region cannot always be clearly recognized. For whatever reasons, the overall efficiency of labeling by either method was rather low.

Carbon, Shenk, and Berg (1975) constructed a better label for poly(A) mapping by cleaving SV40 DNA circles with RI endonuclease, resecting the 5' ends with  $\lambda$ -exonuclease, and polymerizing short poly(dT) on the exposed 3' ends with terminal deoxynucleotidyl transferase. We used some of Carbon's extended SV40 DNA and found it very efficient at labeling poly(A) on Sindbis RNA. The poly(A) stretch was marked where a linear SV40 duplex DNA joined a linear Sindbis single-strand RNA, but the contrast between single strands and double strands in the electron microscope was seldom good enough to pinpoint the SV40 DNA to Sindbis RNA junction. We subsequently noticed that terminal transferase can polymerize poly(dT) at nicks in nicked circular SV40 DNA (as previously reported by Jackson and Berg in a personal communication to Lobban and Kaiser, 1973). We have found that the resulting SV40 circles with poly(dT) tails are excellent markers for poly(A) mapping.

## Results

### SV40-Poly(dT)

When SV40 DNA was incubated with terminal transferase and thymidine triphosphate under the conditions described in Experimental Procedures, the enzyme polymerized long poly(dT) tails on the circle, presumably at single-strand nicks (Figure 1). In trial reactions using an SV40 DNA concentration of 20  $\mu\text{g/ml}$ , the average tail length was 650 bases after 90 min and 2000 bases after 270 min. After the 90 min reaction, there were, on average, two tails per circle. 75% of the treated SV40 DNA bound to poly(A)-sepharose and was eluted in 0.1 M NaCl at about 65°C, the expected melting temperature for poly(A)·poly(dT) duplexes (Riley, Maling, and Chamberlin, 1966). 24 hr incubations did not produce tails longer than about 2000 bases on average, but there were more tails per circle and many unattached pieces of poly(dT). About half the SV40 DNA was in the supercoiled form initially, but almost all the molecules contained poly(dT) after the incubation, so that some endonucleolytic nicking must occur during the terminal transferase reaction.

To prepare SV40 with very short tails, we used a 1 hr incubation with 500  $\mu\text{g/ml}$  SV40 DNA. This produced an average of two visible tails per circle, each about 175 bases long.

### Sindbis RNA

Sindbis RNA was used as a first test system for poly(A) mapping techniques, since the RNA is long and had been studied previously (Hsu et al., 1973). Over 90% of Sindbis molecules contain poly(A), but the size of the poly(A) sequence is somewhat heterogeneous; the average length is about 60 nucleotides, with about 60% of the poly(A) molecules between 50 and 70 bases long (T. Frey and J. Strauss, manuscript in preparation). Sindbis RNA treated with glyoxal was first hybridized with a preparation of SV40 with long (about 2 kb) dT tails. Many clear hybrids were found (Figure 2), but the long tails of poly(dT) were a disadvantage, since the Sindbis RNA often hybridized to the end of the poly(dT). The resolution of these cytochrome spreads is not sufficient to distinguish the poly(A)·poly(dT) duplex region from the single strands, so that the poly(A) end of the Sindbis RNA could be anywhere within a few thousand bases of the SV40. In subsequent experiments, we used SV40 with much shorter tails—about 175 bases long. For the long RNA molecules of Sindbis or RNA tumor viruses, the length of the short poly(dT) tails was negligible, and the poly(A) end of the RNA was assumed to start at the SV40 circle.

In measuring the efficiency of poly(A) hybridization to the SV40-poly(dT), it was not practical to score only full-length (14 kb) Sindbis RNA molecules since very few molecules were unbroken. Of the RNA molecules greater than half size (7 kb), the fraction in clear hybrids with SV40-poly(dT) was only about 40%, even if the RNA was preselected for poly(A) sequences by binding to oligo(dT)-cellulose. It is probable that some of the shorter Sindbis poly(A) sequences would dissociate from the SV40-poly(dT) under our spreading conditions. Breakage of the RNA during the annealing procedure might also lower the apparent hybridization efficiency. Despite the incomplete labeling of Sindbis poly(A), it was clear from these initial studies that the SV40-poly(dT) circles would be effective for mapping poly(A) stretches in tumor virus RNA.

### RD-114

The RNA of the endogenous feline type C virus, RD-114, has been extensively studied in our laboratory (Kung et al., 1975). The 52S RNA from the virion appears to be a dimer (20 kb) whose two 26S subunits (10 kb) are held together in a Y shaped or  $\nabla$  shaped structure that we had previously named the rabbit ears, although we now prefer to describe it as the dimer linkage structure. An additional secondary structure feature observed in this RNA is a loop with a circumference of 3.8 kb located about 2.4 kb from the dimer linkage. The conditions normally used for electron microscope spreading are sufficiently denaturing so that a loop is opened up about 50% of the time.

When the 52S dimer RNA of RD-114 is treated with glyoxal and allowed to hybridize with SV40-poly(dT), the majority of the molecules have structures similar to those shown in Figure 3. The RNA forms a circular structure with the two free ends joined to a single SV40-poly(dT) circle at different (dT) tails and with the dimer linkage structure in the center of the RNA. Thus there is a poly(A) sequence at each of the two free ends of the 52S RD-114 RNA. In the molecule shown in Figure 3A, both loops have opened up; both loops are present in the molecule shown in Figure 3B.

It is rather rare (1 of 67 cases) to see an RD-114 dimer with each end joined to a different SV40-poly(dT) circle (such as is shown in Figure 5 for the RNA from BKD, a baboon virus). This is expected according to the theory of ring closure of linear chains (Jacobson and Stockmayer, 1950; Wang and Davidson, 1966), since the equivalent concentration of the unhybridized poly(A) end in the neighborhood of an end already bound to SV40 is about  $1.4 \times 10^{-8}$  M [assuming a Kuhn statistical segment

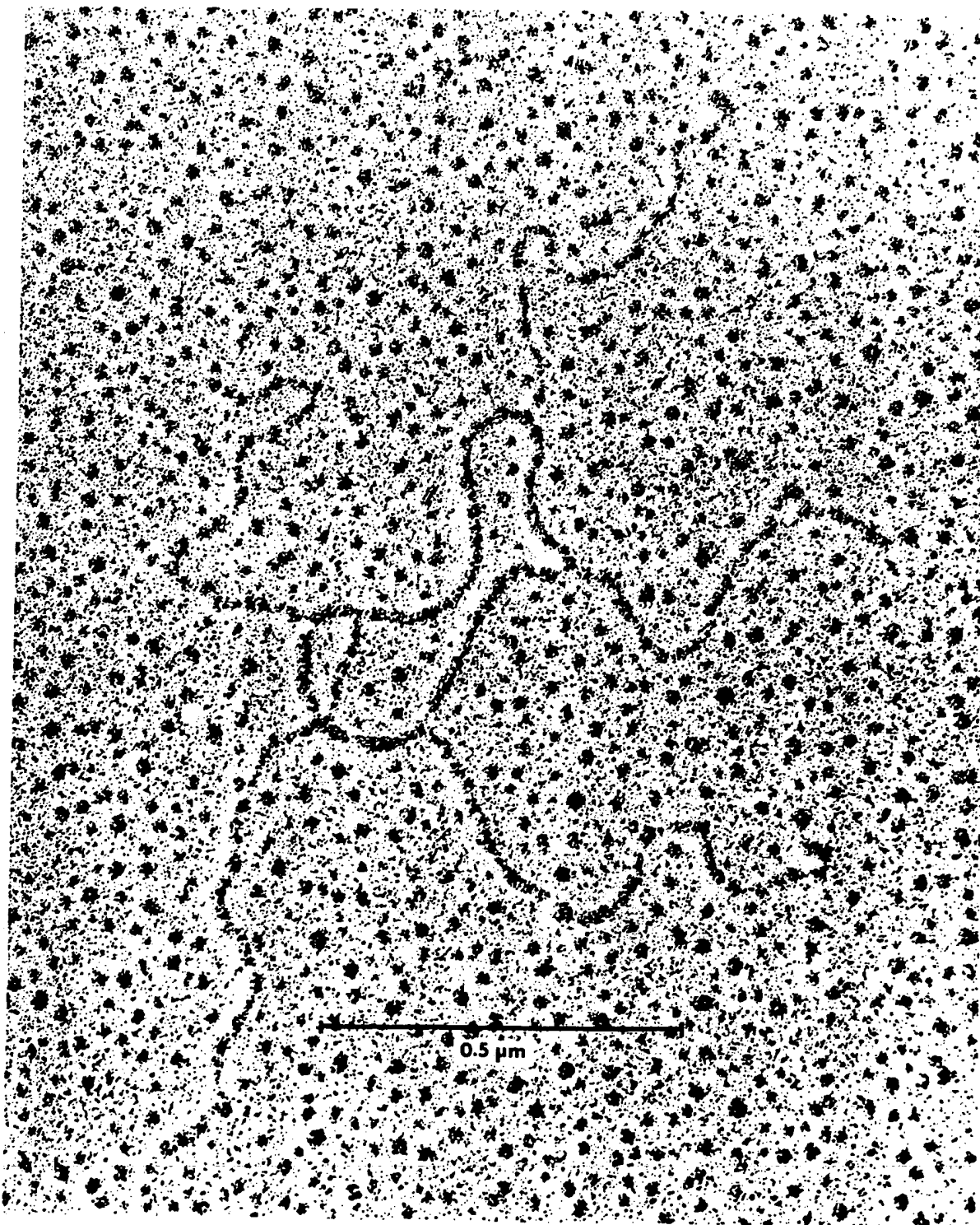


Figure 1. Electron Micrograph of a Molecule of Circular Double-Strand SV40 DNA (Contour Length 5.5 kb) with 5 Attached Poly(dT) Tails  
The tails range in length from 2.2 to 3.0 kb. The molecule was produced by a 4.5 hr terminal transferase reaction with 20  $\mu\text{g/ml}$  of SV40 DNA.



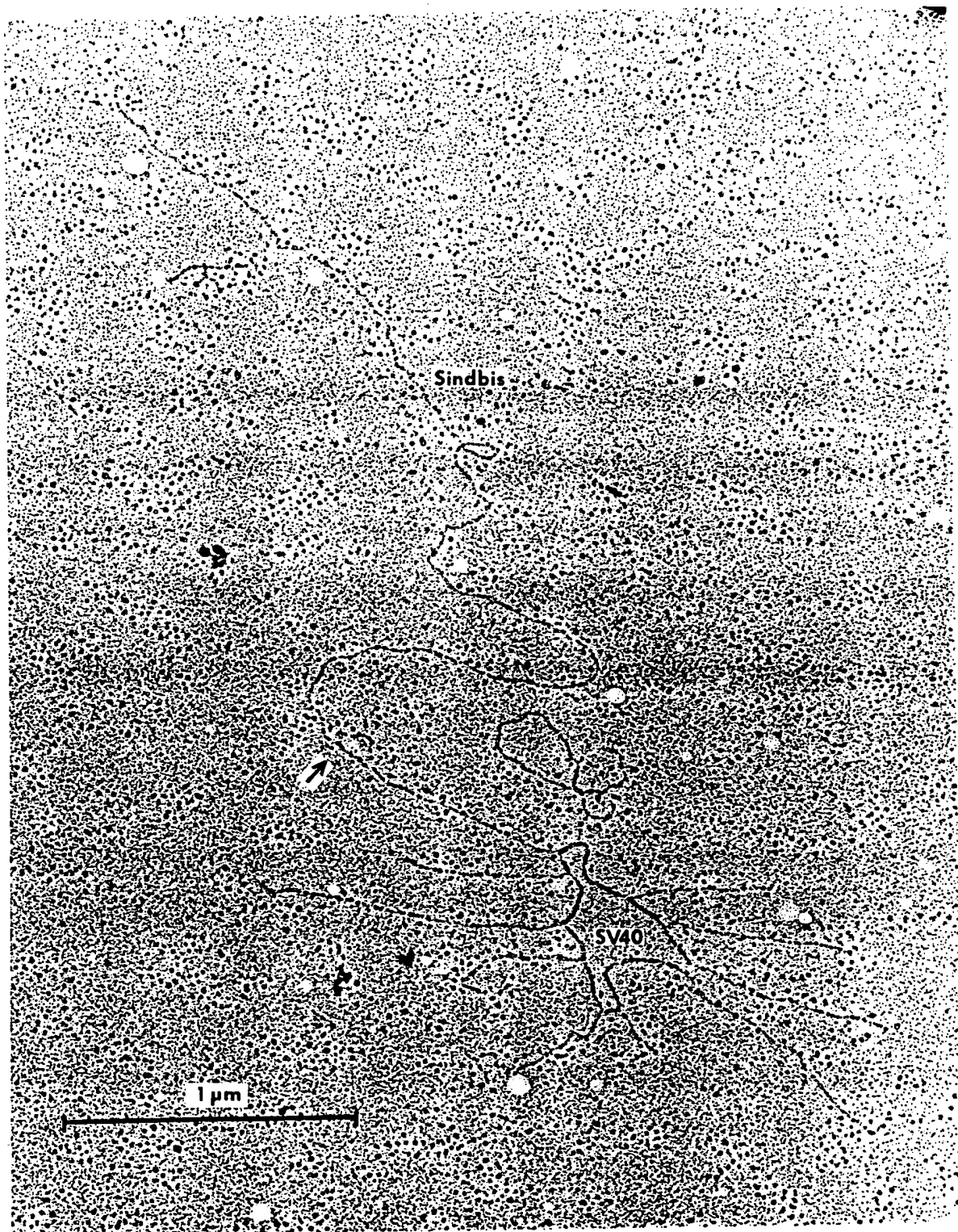


Figure 2. A Molecule of Sindbis RNA Hybridized to an SV40 Circle with Long Poly(dT) Tails  
The arrow indicates the fork that we identify as the junction of the RNA with the poly(dT).

length (b) of  $2 \times 10^{-6}$  cm for RNA (see appendix to Schmid, Manning, and Davidson, 1975)], whereas the concentration of free SV40 molecules is only about  $5 \times 10^{-10}$  M.

If the glyoxal-treated RD-114 is heated to 90°C for 10 sec in 10 mM Tris, 1 mM EDTA before hybridization and spreading, the dimers dissociate at the dimer linkage structure, and the 3.8 kb loops also melt out, leaving 10 kb linear RNA subunits which hybridize to the SV40-poly(dT) at one end only (data not shown). Thus there are no poly(A) sequences buried within the dimer linkage structure.

We wish to count directly the fraction of RD-114 molecules with poly(A) stretches. RD-114 RNA was hybridized with SV40-poly(dT) and spread. A large random sampling of RD-114 molecules was photographed whether or not they were attached to SV40. The length of the RD-114 subunit was determined as the average distance from ends clearly hybridized on SV40 to a clearly defined dimer linkage structure ( $[L] = 9.8$  kb; standard deviation,  $\sigma_s = 1$  kb). All molecules were measured from end to dimer linkage, or from end to end if there was no dimer linkage structure, and those whose lengths were within 1 standard deviation of  $[L]$  were scored (Figure 4). Of these, 76% (88/115) were hybridized to the SV40 circles. This is a minimum estimate of the percentage of monomers with poly(A), because monomer molecules with a few hundred bases at the 3' end broken off would be scored as full length, and because the hybridization efficiency may be <100%. The 76% also gives a minimum estimate for the efficiency of hybridization and scoring with the SV40-poly(dT) circles.

#### Other Tumor Viruses

We have examined the RNAs of several other type C viruses to see if they have similar secondary structure and poly(A) position. BKD, an endogenous baboon virus, contains RNA which after glyoxal treatment shows much the same structure as found with RD-114 (Kung et al., 1976). Again, we see a dimer linkage structure and 3.7 kb loops, 1.9 kb from the dimer linkage. The dimer linkage structure itself is slightly different, usually looking like  $\nabla$  or  $+$  or sometimes  $\chi$ . Hybrids with SV40-poly(dT) again show poly(A) regions at both free ends (Figure 5).

WoMV is a simian sarcoma virus isolated from a woolly monkey fibrosarcoma. Some investigators refer to this virus preparation as SSV-1. It should be noted that WoMV preparations contain a large excess of nontransforming helper virus (Wolfe, Smith, and Deinhardt, 1972), and so it is really the RNA of this associated virus that we are studying here. The usual glyoxal treatment dissociated most dimers into subunits, indicating that the cohesion

of the WoMV monomers within the dimer linkage structure is less stable than for RD-114 or BKD. Nevertheless, many dimers were observed and measured. There is a dimer linkage structure, usually  $\nabla$  or  $+$  shaped, and loops of about 3.9 kb, 1.4 kb from the dimer linkage (Kung et al., 1976). Both free ends hybridize to SV40-poly(dT) (Figure 6).

For Rous sarcoma virus, the usual 1 hr incubation with glyoxal causes dissociation of all of the 60-70S RNA to 9.5 kb monomers, indicating that the 60-70S complex dissociates more readily than does the WoMV dimer. It is difficult to find spreading conditions under which the RNA is sufficiently extended to be traced while still retaining structures larger than 9.5 kb monomers. The best results so far have been obtained by treating 60-70S RNA with glyoxal for only 10-15 min, dialyzing against 10 mM Tris, 1 mM EDTA as usual, heating that low salt solution to 37°C for 15 sec, and then hybridizing and spreading. This procedure, like the gene 32 spreading method of Mangel, Delius, and Duesberg (1974), gave some molecules with a total contour length of about 19 kb. Figure 7 is a plot of the length distribution of all molecules in a particular sample which were attached to an SV40-poly(dT) label. There is a clear peak at the monomer RNA length of about 9.5 kb. Of the molecules that are of dimer length (about 19 kb), about half are attached to SV40-poly(dT) circles by two poly(A) ends (solid bars). In these molecules, the structures are often not easily interpretable, and the non-poly(A) ends are usually not closely associated. Such a molecule is shown in Figure 8a. We do, however, find rare examples in which the two non-poly(A) ends of the RSV monomers are joined in an apparent dimer linkage structure (Figure 8b).

#### Discussion

The method described here should be generally useful for mapping sufficiently long poly(A) sequences at the ends of single-strand RNA molecules. The efficiency of labeling is high for the poly(A) ends on Sindbis RNA and for the several tumor virus RNAs studied, although there is only a slight excess of SV40-poly(dT) molecules over viral RNA molecules in the hybridization mixture. The double-strand SV40 circles stand out clearly in the electron microscope; this permits rapid scanning for hybrids at low magnification. Since the single strand of the hybridized RNA stops right at the double-strand circle, there are very few accidental overlaps that would be scored as hybrids, and the positioning of the end is limited only by the size of the poly(dT) tails. In addition, the SV40 circle provides an automatic internal length standard. This marker should be very useful for map-

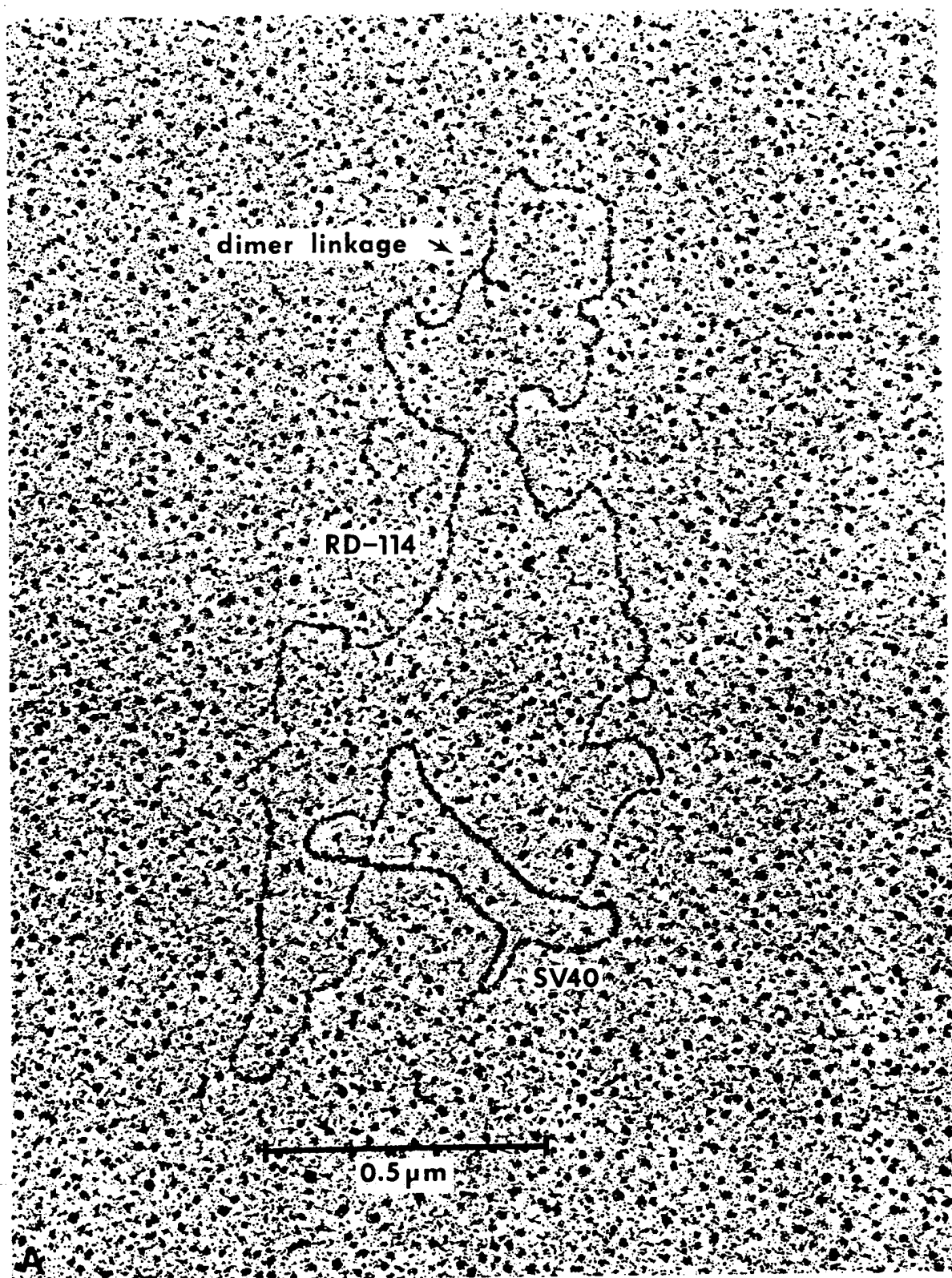
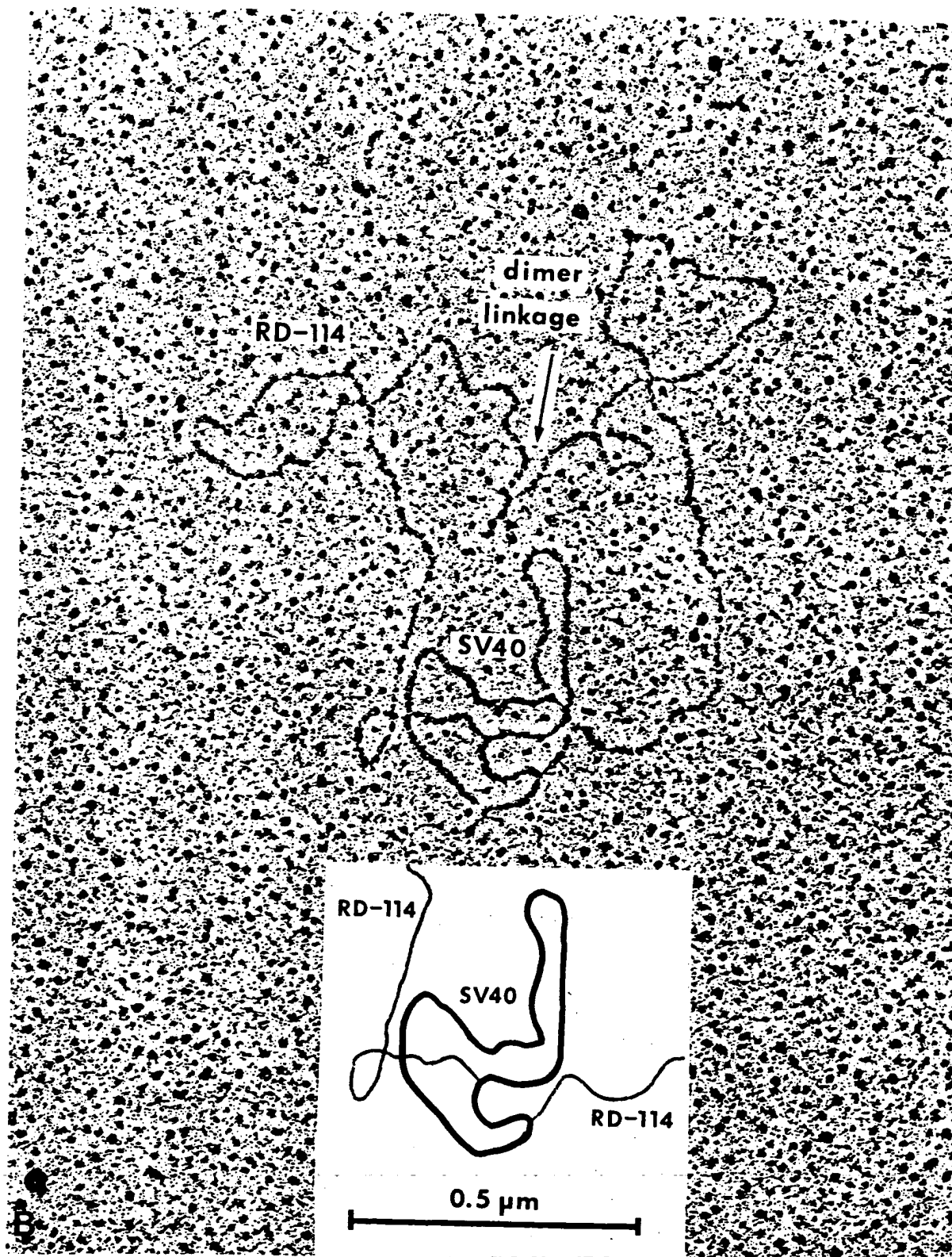


Figure 3. An RD-114 RNA Dimer without Loops (A) and with the Symmetrically Disposed Loops (B), Each Hybridized at Both Ends to an SV40 DNA Circle with Short Poly(dT) Tails  
The dimer linkage structure is indicated. A tracing of the connections of the RNA with the SV40 circle is shown for (B).



ping secondary structure features or heteroduplex regions with respect to the poly(A) ends of viral RNAs, mRNAs, and heterogeneous nuclear RNAs. However, the technique relies on glyoxal treatment and low formamide spreading; the hybrids are not stable in 50% formamide.

We do not know how the efficiency of labeling varies as a function of the poly(A) length, but we suspect that poly(A)·poly(dT) hybrids shorter than about 50 base pairs would dissociate in 30% formamide. The present method will probably be poor for mapping internal poly(A) sequences within long single-strand molecules, since hybrid SV40 circles would often lie on top of the single-strand molecule. There would then be two or more crossover points, and it would not be clear which intersection is the poly(A) site.

Many oncornavirus RNAs have been shown to contain poly(A) (Lai and Duesberg, 1972; Green and Cartas, 1972; Ihle, Lee, and Kennedy, 1974; Wang and Duesberg, 1974; Quade, Smith, and Nicols, 1974), and in some cases (including RSV), the end of the poly(A) stretch has been identified as a 3' end (Wang and Duesberg, 1974; Quade et al., 1974; Rho and Green, 1974). In RSV, it appears that there are only two subunits per 60–70S complex by biophysical (King, 1976) and electron microscopic measurements (Mangel et al., 1974; these

results), and all 35S subunits are probably identical (Duesberg et al., 1974; Weissmann et al., 1974). The studies reported by Kung et al. (1975, 1976) show that RD-114, BKD, and WoMV viral RNAs all have a dimer structure with two monomer ends joined in the dimer linkage structure. The present study shows that each monomer has a poly(A) stretch on the free end. Assuming that RD-114, BKD, and WoMV have identical subunits with poly(A)s at the 3' ends, then the dimer linkage structure must be a complex of two identical (not homologous) 5' ends. Some speculations as to the nature of the binding of the monomers in the dimer linkage structure are given in the accompanying paper (Kung et al., 1976).

Only a few dimer molecules with overlapping 5' ends were observed in Rous sarcoma virus RNA, and there were no reproducible large loops. It may be that the 5' to 5' dimer linkage structure and the loops occur in native RSV 60–70S RNA, but that they are much more readily dissociated than for the other RNAs studied here. Alternatively, the few structures we have seen may be due to accidental overlaps.

There have been reports that not all viral subunits have poly(A) ends; only 65% of RSV subunits (Wang and Duesberg, 1974) and 40% of RD-114 subunits (Ihle et al., 1974) bound to oligo(dT)-cellulose or poly(U)-sepharose. In these several studies, however, the full-size subunits were purified by sucrose gradient sedimentation, and this procedure depends critically upon the resolving power of the gradient and on the quality of the RNA (that is, what fraction of the molecules are almost, but not quite, full-length). King and Wells (1976) have recently shown that when RSV is collected with very short (10 min) harvests and the RNA subunits are resolved on polyacrylamide gels, >90% of the subunits contain poly(A). We have shown here by direct counting of hybrids that at least three fourths of the intact RD-114 subunits have poly(A). Our ability to resolve full-length molecules from almost full-length is limited by the 10% variation in length of glyoxal-treated RD-114 RNA. Furthermore, as shown by King and Wells (1976) and many other investigators, the quality of the RNA can probably be improved by harvesting the virus at shorter intervals. Our results, therefore, tend to support the view that all intact subunits of RD-114 have poly(A) ends.

#### Experimental Procedures

SV40 DNA was a gift from Dr. Harumi Kasamatsu. It was extracted from SV40-infected TC-7 African Green Monkey cells. Sindbis virus, grown on chick embryo fibroblasts, was a gift from Dr. Jim Strauss, and the RNA was isolated as described previously (Hsu et al., 1973). RD-114, BKD, and WoMV viruses were prepared as described in the accompanying paper (Kung et al., 1976). RSV

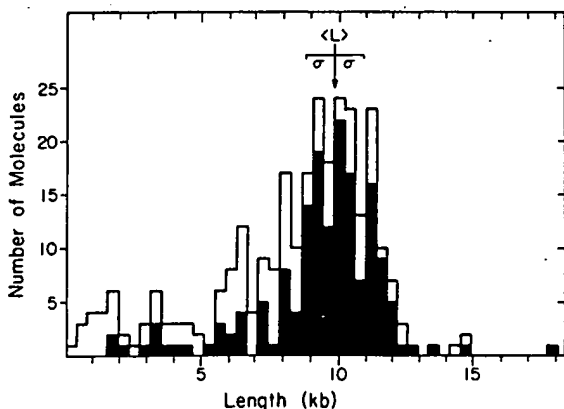


Figure 4. A Measurement of the Fraction of Full-Length Monomer Units Having Poly(A) Ends Attached to SV40-Poly(dT)

All long molecules in fields chosen at random were photographed. Lengths of monomer subunits were measured. The glyoxal treatment caused dissociation of about 25% of the RD-114 RNA into monomer-length molecules with no recognizable dimer linkage structure. These were measured directly. In molecules with a clear dimer linkage structure, the length from this feature to each end was measured. Open bars represent all molecules; solid bars represent molecules hybridized to SV40-poly(dT). The indicated average monomer length ( $\langle L \rangle$ ) and standard deviation ( $\sigma$ ) were calculated from molecules which had a dimer linkage structure and which were attached to SV40-poly(dT), and thus must have been unbroken monomer subunits. Of molecules within 1 standard deviation of this average full length, 76% are attached to SV40-poly(dT).



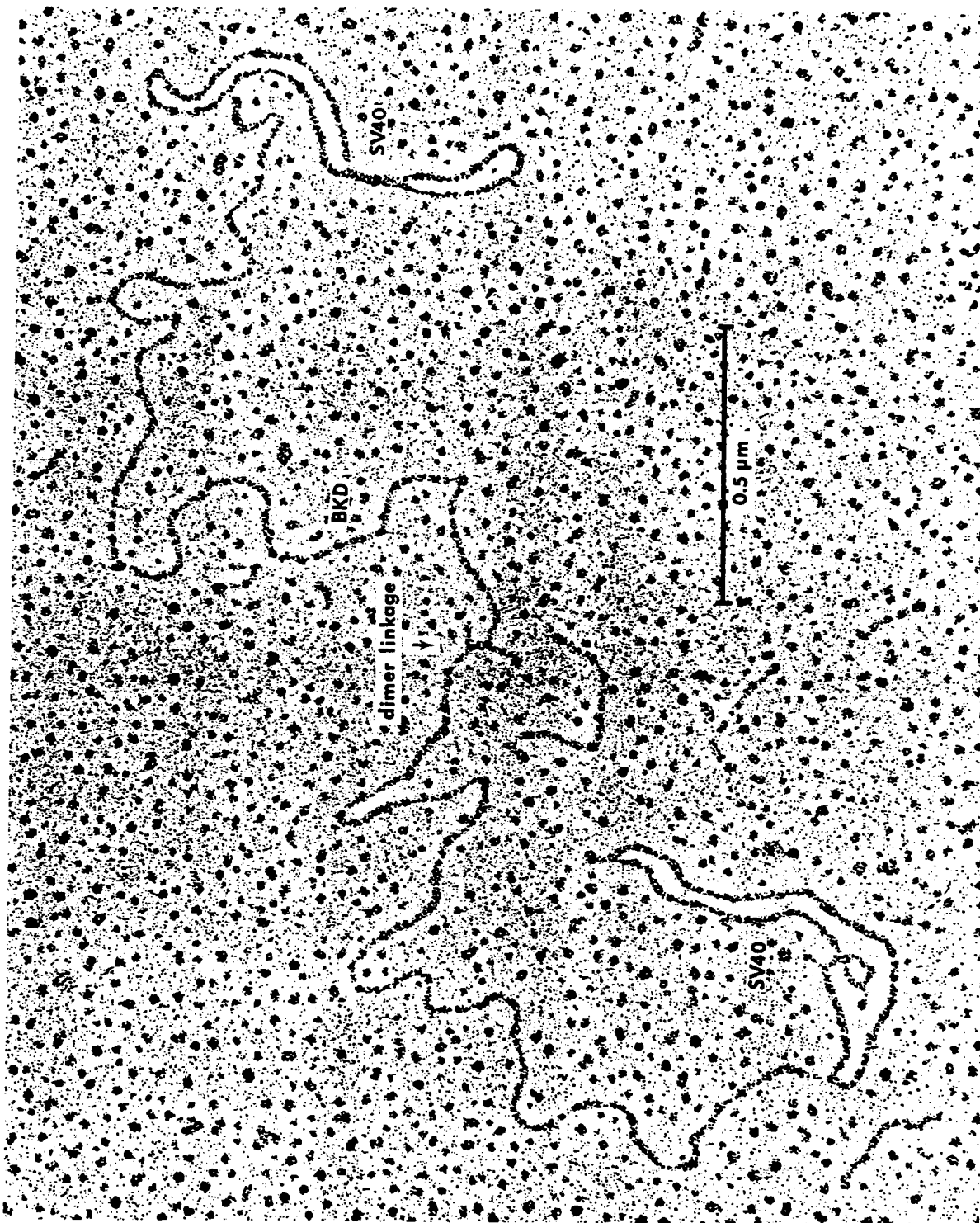


Figure 5. A BKD RNA Dimer Hybridized to SV40 Circles with Short Poly(dT) Tails

An arrow indicates the dimer linkage structure. This molecule is one of the unusual cases in which each poly(A) end is hybridized to a different SV40-poly(dT) circle.

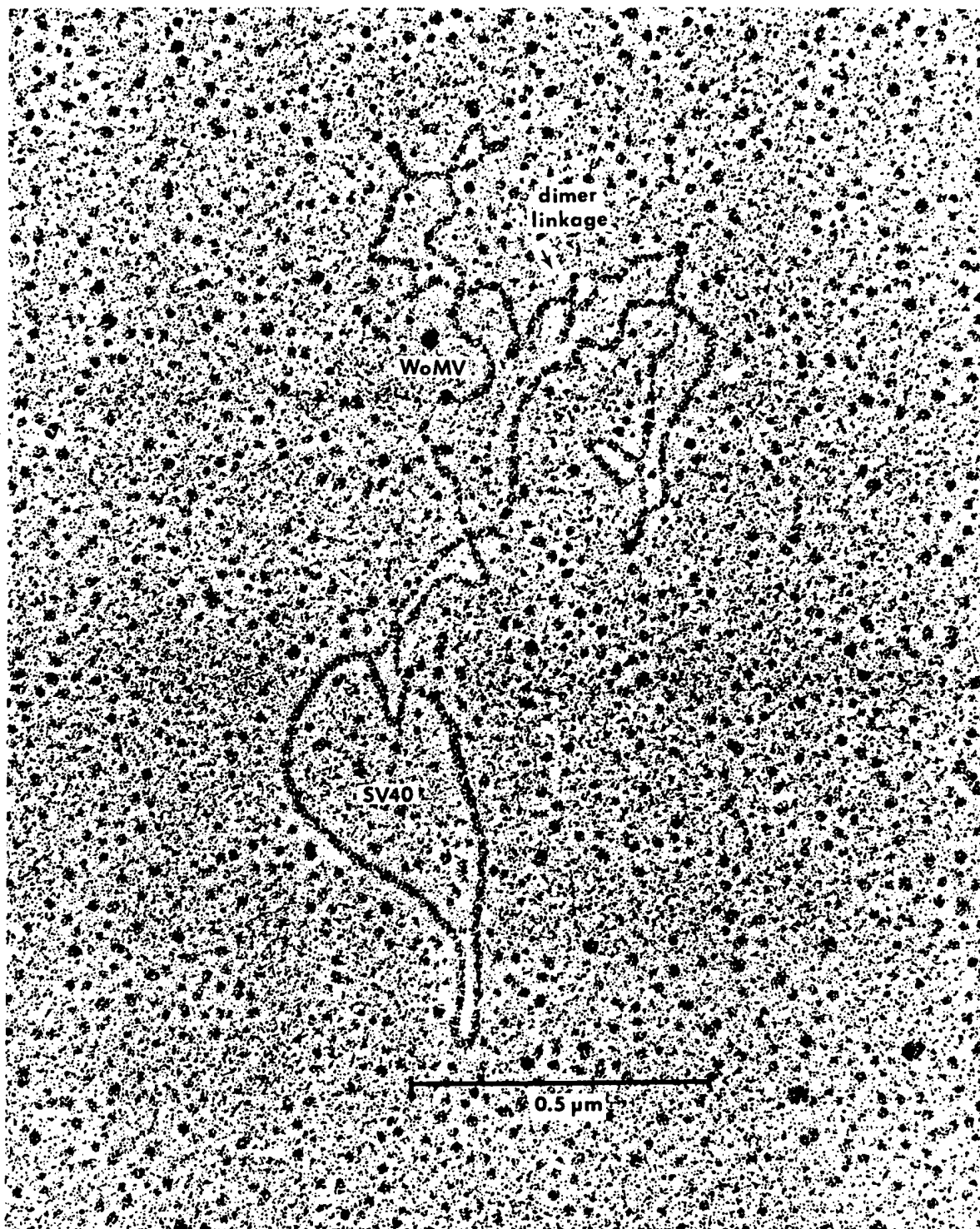


Figure 6. An WoMV RNA Dimer Hybridized to an SV40 Circle with Short Poly(dT) Tails  
The RNA shows the symmetrically placed large loops. The dimer linkage structure is indicated.

(Rous sarcoma virus, Prague C. strain), grown in chick embryo fibroblasts, was obtained from Dr. Peter Vogt. All type C viruses were collected at 3 h intervals, and the cell culture medium containing the virus was frozen and stored at  $-70^{\circ}\text{C}$ . The viruses were banded in sucrose, the RNA was phenol-extracted and ethanol-precipitated, and the 60–70S RNA was isolated by sedimentation as described previously (Kung et al., 1975). Terminal deoxynucleotidyl transferase, purified from calf thymus, was purchased from P & L Biochemicals, Inc. Oligo(dT)-cellulose, type T-2, was pur-

chased from Collaborative Research, Inc. To minimize ribonuclease contamination, glassware was baked overnight at  $150^{\circ}\text{C}$ , and water was double-distilled.

#### Preparation of SV40-Poly(dT)

SV40 DNA (a mixture of supercoiled and nicked circular DNAs) was extended with terminal transferase in a reaction mix of 10–50  $\mu\text{l}$  containing 0.1 M  $\text{KH}_2\text{PO}_4$  and 0.05 M cacodylic acid adjusted to pH 7.0 with KOH, 1 mM  $\beta$ -mercaptoethanol, 1 mM  $\text{CoCl}_2$ , 3 mM thymidine triphosphate (TTP), 500 units per ml terminal transferase, and up to 0.5 mg/ml SV40 DNA. The solution was incubated at  $37^{\circ}\text{C}$  for 1–24 hr, depending upon the tail length desired. The reaction mixture was then diluted about 5 fold into 10 mM Tris, 1 mM EDTA (pH 8.5), and layered onto a gradient of 5–20% sucrose in the same buffer. Sedimentation (SW50.1 rotor, 44K rpm, 6 hr,  $4^{\circ}\text{C}$ ) separated the SV40-poly(dT) from unincorporated TTP and unattached poly(dT). The SV40-poly(dT) was collected and dialyzed against 10 mM Tris, 1 mM EDTA (pH 8.5), and stored at  $-70^{\circ}\text{C}$ . Sometimes the reaction was run using  $^3\text{H}$ -labeled TTP (10 Ci/mole), in which case incorporation could be measured by acid precipitation, or using  $^3\text{H}$ -labeled SV40 DNA ( $3.6 \times 10^5$  cpm/ $\mu\text{g}$ ), so that the fraction of circles extended could be measured by fractionation on poly(A)-sepharose. However, SV40-poly(dT) used for electron microscope labeling was not fractionated on poly(A)-sepharose.

#### Poly(A)-Sephadex

Poly(A)-sephadex was prepared by the CNBr coupling method of Berridge and Aronson (1973) using 200 mg CNBr/ml packed se-

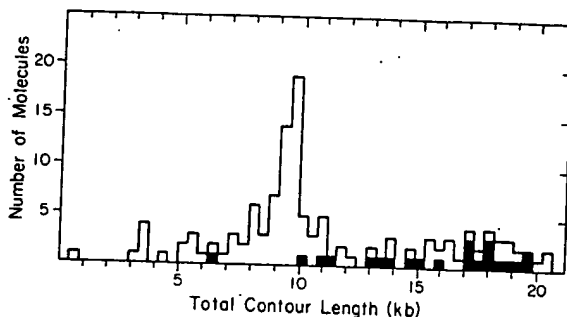


Figure 7. Histogram of Total Contour Lengths of Randomly Selected RSV RNA Molecules Which Are Attached to SV40-Poly(dT)

Open bars represent all molecules; solid bars indicate molecules attached to SV40 by two separate ends. There were no molecules found attached to SV40 by three or more ends.

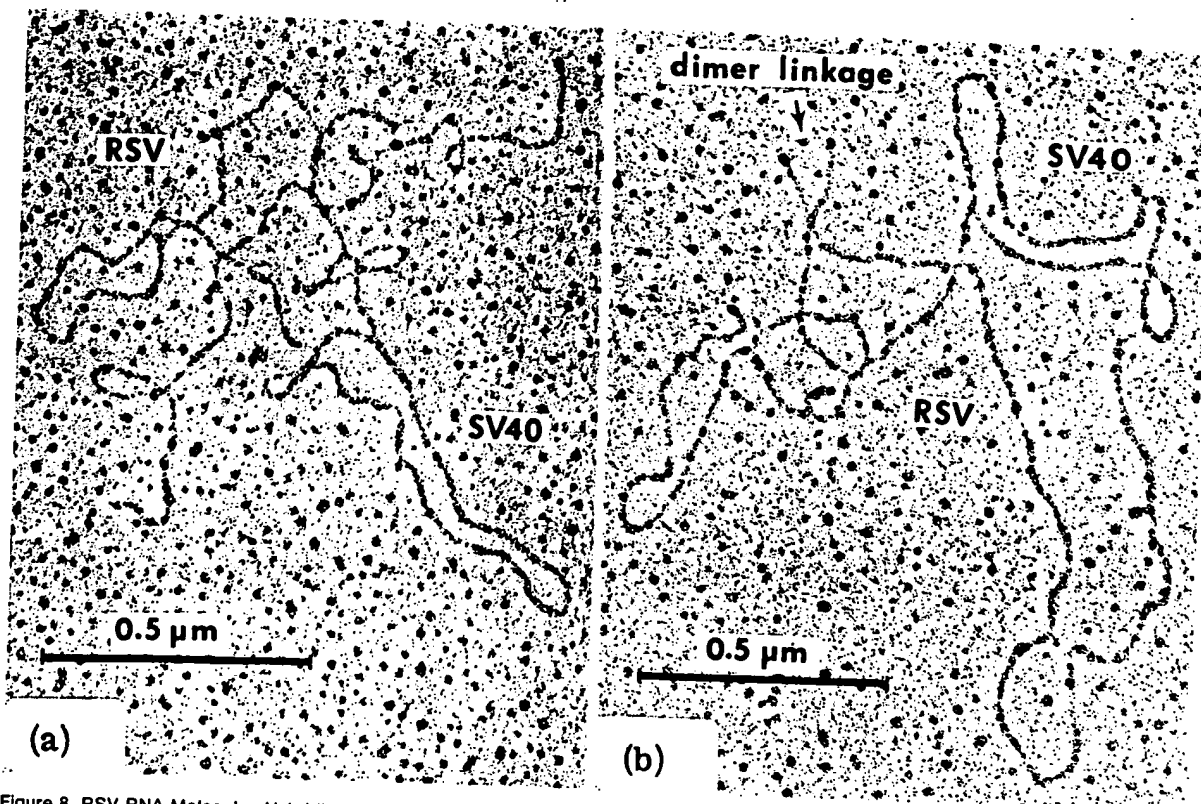


Figure 8. RSV RNA Molecules Hybridized to SV40 Circles with Short Poly(dT) Tails  
The arrow in (b) indicates a presumed dimer linkage structure.



pharose. The sample to be absorbed to the poly(A)-sepharose was mixed with 1 ml of 0.1 M NaCl, 10 mM Tris, 1 mM EDTA (pH 7.5), and put over a column of 1 ml bed volume at a rate of about 0.2 ml/min at 25°C. The column was eluted with the same buffer at increasing temperature.

#### Oligo(dT)-Cellulose

The oligo(dT)-cellulose column (1 ml bed volume) was loaded and washed as the poly(A)-sepharose, except that everything was done at 4°C. The column was eluted at 37°C with 5 ml of 10 mM Tris, 1 mM EDTA (pH 7.5) at a flow rate of 1 ml/min.

#### Glyoxal Treatment and Hybridization

RNA was incubated with 1 M glyoxal, 10 mM sodium phosphate (pH 6.7) at 37°C for up to 1 hr. The mixture was then dialyzed against 10 mM Tris, 1 mM EDTA (pH 8.5) for 24 hr at 4°C to insure removal of glyoxal reversibly bound to A and C residues (Broude and Budowsky, 1971). About 0.01 µg of RNA was then mixed with about 0.02 µg SV40-poly(dT) in 5–20 µl of 0.4 M Tris, 0.04 M EDTA (pH 8.5). The mixture was allowed to stand at room temperature for 5–15 min before spreading. The expected  $Cot_{1/2}$  for poly(A)<sub>200</sub>·poly(dT)<sub>200</sub> reassociation at a cation concentration of 0.25 M at 38°C is about  $2 \times 10^{-4}$  M sec (Lee and Wetmur, 1972). Our incubations [about 100 ng/ml in poly(A) and poly(dT), about 0.24 M in cations, 10', 25°C] reach a  $Cot$  of about  $1.8 \times 10^{-4}$  M sec, that is, a 90 fold excess over  $Cot_{1/2}$  [neglecting corrections for temperature and for the unknown effects of the lengths of strands to which the poly(A) and poly(dT) are attached].

#### Spreading

Hybrids were spread by the formamide, cytochrome C method (Davis, Simon, and Davidson, 1971) using 30 or 40% formamide in the hyperphase with 2 or 12% formamide in the hypophase, respectively. Grids were rotary-shadowed with platinum palladium and examined using a Phillips 300 electron microscope. Molecules of interest were photographed and the contour lengths were measured using a Hewlett-Packard digitizer. A diffraction grating was used to calibrate the length of SV40 circles ( $1.76 \pm 0.06 \mu\text{m}$ ), and SV40 was available as an internal length standard on all spreadings. Kilobase lengths of glyoxal-treated single-strand RNA were computed assuming 3.91 kb/µm as measured for glyoxal-treated *E. coli* 23S ribosomal RNA (Hsu et al., 1973). The lengths of RD-114 and RSV monomers given here are slightly larger than those previously reported (Kung et al., 1974, 1975). This correction resulted from a more accurate calibration of the microscope magnification.

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We are grateful to John Carbon for his advice and suggestions about the general method of using poly(dT) tails on duplex DNA as a label. We have profited from the counsel of our co-workers, Sylvia Hu, Hsing-Jien Kung, James Bailey, Margery Nicolson, Robert McAllister, and Peter Vogt.

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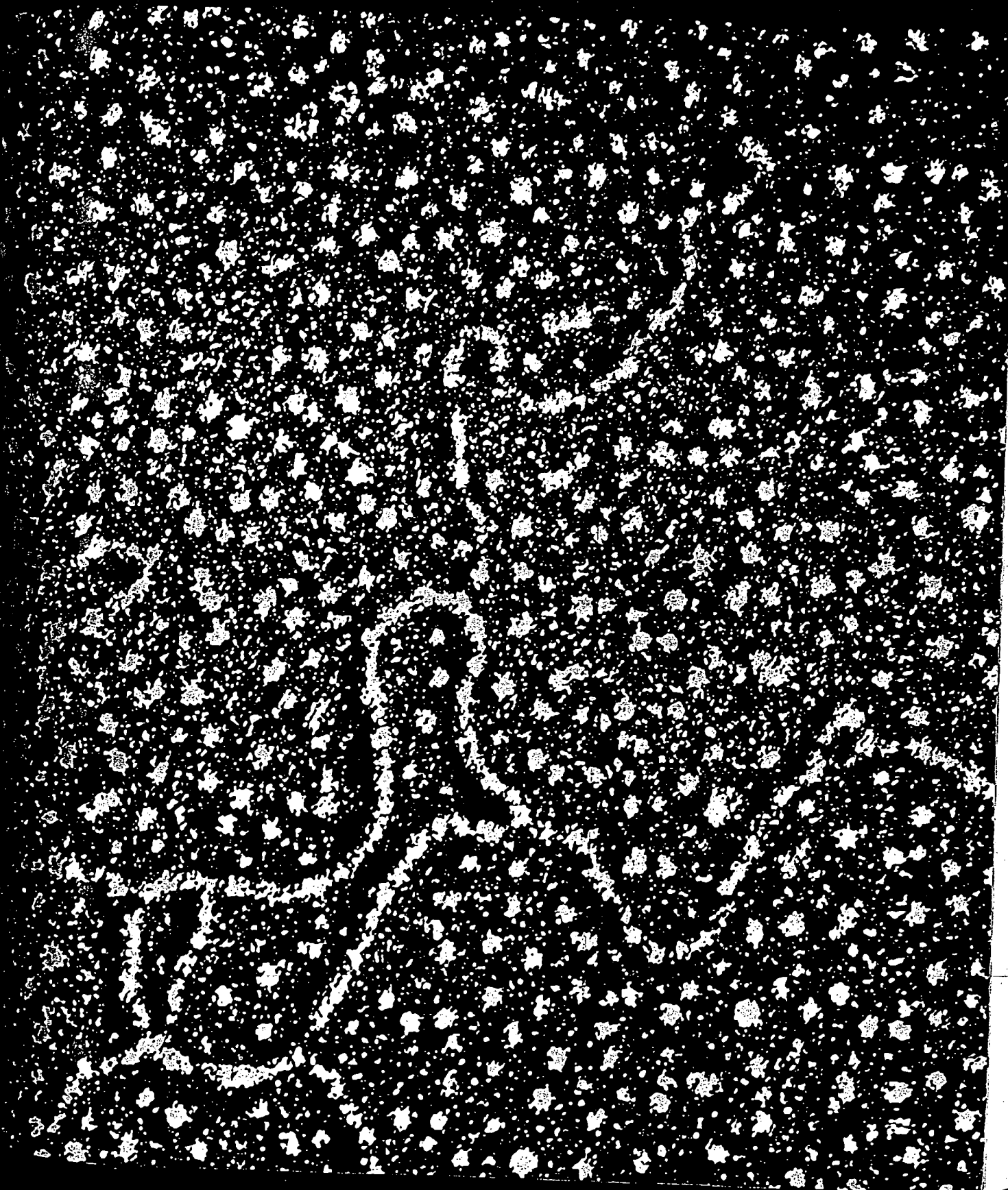
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# Fine structure mapping of an avian tumor virus RNA by immunoelectron microscopy

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## ABSTRACT

The RNA of a deleted strain (lacking Src gene) of an avian sarcoma virus (ASV) was examined by a newly developed immunoelectron microscopic procedure which uses anti-nucleotide antibodies as probes. After denaturation of the RNA and reaction with a high affinity, highly specific anti-7-methylguanosine-5'-phosphate (anti-pm<sup>7</sup>G), 81% of 106 molecules examined were found to have antibody at one terminus, in agreement with the presence of a pm<sup>7</sup>G cap in ASV-RNA. Hapten inhibition by pm<sup>7</sup>G could be demonstrated. Experiments with anti-A and with anti-poly A gave results consistent with the known structure of ASV-RNA. In particular the presence of a 3' poly A tail. These studies illustrate the feasibility of using anti-nucleotide antibodies in a combined immunochemical and electron microscopic study of the fine structure of nucleic acids.

## INTRODUCTION

This laboratory has been using anti-nucleotide antibodies<sup>1</sup> for the study of nucleic acids, chromatin and polytene and metaphase chromosomes (reviewed in ref 2). The studies on chromosomes were of an immunocytological nature and led to information on the location and organization of AT- and GC-rich clusters, as well as clusters of 5-methylcytosine. For studies on human metaphase chromosomes, procedures adaptable to the electron microscope were developed<sup>3</sup> and details of the arrangement of 5-methylcytosine residues near the centromeres of chromosomes 1, 9, 15, 16 and the Y could be seen.

We have now succeeded in developing procedures that permit utilization of the highly specific anti-nucleotide antibodies in electron microscopic studies of what might be called the fine structure of strands of nucleic acids. The technique is not intended as a substitute for sequencing but has attributes

at 4  $\mu$ g/ml to prevent RNase cleavage. No cleavage was detectable after 3 months of storage at 4°C.

Characteristics of the antibodies - Anti-pm<sup>7</sup>G was prepared as described earlier<sup>7</sup>. A globulin fraction was specifically purified by adsorbing and eluting from pm<sup>7</sup>G-Affigel beads as follows: A 500  $\mu$ l volume of a 10% suspension of pm<sup>7</sup>G-Affigel beads (Bio-Rad Affigel 703 hydrazide acrylamide beads) derivatized with pm<sup>7</sup>G (P-L Biochemicals)<sup>7</sup> was incubated at 4°C for 24 hours on a slowly rotating pinwheel with 1 ml of the rabbit anti-pm<sup>7</sup>G-BSA globulin fraction in a 1.5 ml Brinkmann polypropylene tube. The incubated beads were washed three times with 1 ml of saline, each wash followed by centrifugation. The final pellet was resuspended in 500  $\mu$ l of 0.2M HCl-glycine buffer, pH 2.6, incubated 15 min at 4°C and pelleted again at 4°C. The supernatant was withdrawn, immediately neutralized with 100  $\mu$ l 1M K<sub>2</sub>HPO<sub>4</sub>, and dialyzed in a collodion bag (Schleicher & Schuell) against saline at 4°C. The OD<sub>280</sub> after dialysis was 0.37 or 270  $\mu$ g of antibody per ml. This procedure normally yields about 50% of the binding activity of an equal volume of the globulin fraction, as determined by radioimmunoassay (below). RNase activity was undetectable after affinity purification as measured by TCA precipitation of <sup>3</sup>H-poly C<sup>7</sup>. Radioimmunoassay of anti-pm<sup>7</sup>G - In 1.5 ml Brinkmann polypropylene tubes, 5  $\mu$ l of affinity-purified anti-pm<sup>7</sup>G was incubated with 10  $\mu$ l of inhibitor and 5  $\mu$ l of [<sup>3</sup>H]-pm<sup>7</sup>G (NEN, 5800 cpm, spec. act. 13.2 Ci/mole, diluted in PBS) for 1.5 hours at 37°C. PBS (4 ml) was added and the solution was then filtered on a manifold with nitrocellulose filters (Gelman 0.45  $\mu$ m pore size, prewet with PBS) and washed once with 1 ml of PBS. The filters were dried and counted in 10 ml Hydrofluor (National Diagnostics). About 18% of the input counts were bound by antibody without inhibitor. All assays were run in duplicate.

The purity of the inhibitors, GMP (Sigma), AMP (Sigma), pm<sup>7</sup>G (P-L Biochemicals) was assessed by PEI-cellulose chromatography (PEI impregnated plastic sheets 0.1mm cellulose MN 300) developed in 0.4M NH<sub>4</sub>HCO<sub>3</sub> at room temperature. One spot for each was found under short-wave UV; pm<sup>7</sup>G was fluorescent.

Anti-A and anti-C were prepared<sup>1</sup> and specifically purified<sup>9</sup>. Radio-

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of its own that should be useful in structural studies. First of all, the anti-nucleotide antibodies react only with nucleosides or nucleotides that are in single stranded regions of nucleic acids, i.e. regions in which there is no base pairing. Therefore, the antibodies can be used as direct probes of nucleic acid conformation; much as S1 nuclease is used<sup>4</sup>. Second, the antibodies have been shown to be highly specific. For example, antibodies to 5-methylcytosine<sup>5</sup>, to 6-methyladenine<sup>6</sup> and to 7-methylguanosine-5'-phosphate<sup>7</sup> show only minor cross reactions with the homologous unmethylated bases. The availability of highly specific monoclonal anti-nucleotide antibodies will further enhance the value of immunoelectron microscopic procedures, and, to this end, we have recently isolated hybridomas that synthesize highly specific anti-5-methylcytosines which, by passive hemagglutination, show no reaction with cytosine. Thus, the procedure described in this paper can be a rapid reliable way of locating methylated bases along the chain of nucleic acids.

In this paper, we describe the development of procedures for the immunoelectron microscopic study of a transformation defective (td) avian sarcoma virus (ASV). The virion RNA is predominantly a 70S structure containing 2 identical subunits. The RNA of each subunit of the non-defective virus has about 9,500 nucleotides, with a 7-methylguanosine "cap" at the 5' end and a poly A tail of about 200 residues at the 3' end. It also has a number of 6-methyladenosine residues scattered throughout the genome. The strain we are examining (Bratislava 77) is transformation defective (td), and lacks an 1800 residue segment in the region of the *src* gene. (For a good review, see ref. 8.) We have studied the reaction of the 35S subunit with anti-7-methylguanosine-5'-phosphate (anti-pm<sup>7</sup>G) of high specificity and affinity<sup>7</sup> and have strong evidence for the visualization of the 7-MeG "cap." Using anti-A, anti-C and anti-poly A, we have preliminary evidence for visualization of the poly A tail and A-rich regions within the RNA strand.

#### EXPERIMENTAL

The 35S RNA subunit of transformation defective (td) Bratislava 77 strain, an avian tumor virus (ASV), was the gift of Dr. R.V. Guntaka. It was stored on ice in 99.5% deionized formamide, 1mM EDTA, pH 7.5,

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immunoassay. Ouchterlony and tube precipitation assays showed specificity for the respective purine and pyrimidine bases. Anti-poly A was prepared according to Kahana and Erlanger<sup>10</sup>. It was specifically purified by R. Pohlman using a poly A-Sepharose immunoabsorbent.

Electron microscopy - A modification of the procedure of Vollenweider et al.<sup>11,12</sup> was used. The detergent spreading agent (BAC-Form) was stored at room temperature. It was prepared by dissolving an equal amount by weight of C<sub>14</sub> and C<sub>16</sub> benzyltrimethylammonium chloride (Fluka) at a total concentration 0.02% in 100% formamide.

Grids were coated with a 1:1 mixture of 3.5% parlodion<sup>13</sup> and butyl methacrylate in collodion (CAM)<sup>14</sup>. The CAM-parlodion grids were thinner and stronger than parlodion alone and provided better contrast in our experiments. Just before use, dried grids were floated on a  $3 \times 10^{-3}\%$  solution of ethidium bromide in H<sub>2</sub>O for 10 mins and then washed for 10 mins in H<sub>2</sub>O (Szybalski, personal communication). The 10 minute exposure to ethidium bromide gave more uniform spreading consistency, despite variations of 6° in temperature and 15% in relative humidity during the year. After specimens were applied (below), the grids were given a final H<sub>2</sub>O wash of 2 mins, and the samples were dehydrated by immersion in a 90% methanol wash for 30 seconds and air-dried<sup>15</sup>. Methanol did not lengthen the molecules in our case but did eliminate the small discontinuities apparent after ethanol dehydration.

Grids were rotary shadowed with platinum-palladium at an angle of 7° and examined in a Hitachi HU-11C transmission electron microscope at 75KV and at a magnification of about 20,000. A line grating replica (Fullam, 2160 lines/mm) was photographed with each series of micrographs, keeping the intermediate lens current constant for the series. Micrographs were projected at a 5.4X magnification. Total magnification was usually about 112,500. Molecules and antibody were traced on paper. Nucleic acid molecular lengths and positions of antibody were measured with a Numonics Graphics Calculator. In addition,  $\phi$ X174 am3CS70 single-stranded virion DNA (Biolabs) spread under the same conditions as ASV (below) was used as an external length standard; it is 5386 nucleotides long<sup>16</sup>.

In measuring the location of antibody on nucleic acid strands, the

centers of the antibody molecules were considered the attachment site.

ASV molecules and attached antibodies were measured several times and in both directions. The deviations were random averaging 0.09 cm, which corresponds to 0.008  $\mu$ m at final magnification.

Preparations of specimens - A solution (100  $\mu$ l) containing 10 ng of ASV,  $7 \times 10^{-4}\%$  BAC, 56% of 99.5% deionized formamide-1mM EDTA (pH 7.5) and 44% 10mM triethanolamine (TEA)-1mM EDTA (pH 8.0) was applied as a drop to a parafilm surface in an atmosphere of at least 80% relative humidity. After 11 mins, a grid was touched to the surface of each drop. Less than 2 mins before reaction with the RNA on the grid, 1  $\mu$ g of antibody (approximately 0.3 mg/ml) was diluted with 4.0 ml of 10mM TEA-1mM EDTA - pH 8.0 ("Buffer") and applied to a parafilm strip in 500  $\mu$ l drops. Grids touched to ASV solutions were floated on the drops for 8 mins followed by a water wash and dehydration. The conditions for reaction with all of the antibodies were the same. For the inhibition studies, 0.25  $\mu$ g of antibody was diluted with 100  $\mu$ l of an 0.1% solution of the appropriate monophosphate nucleoside in "Buffer" adjusted to pH 7.5 and kept cold in ice for at least 24 hours. Immediately prior to use, it was diluted with 900  $\mu$ l of "Buffer." The subsequent procedure was the same as for uninhibited antibody. Higher final concentrations of nucleotides caused aggregation of the nucleic acids; lower did not inhibit as well. Antibody-inhibitor reaction times of 1-2 hours were never as effective as 24 hours or more.

The control grids, i.e. ASV alone or  $\phi$ X174 alone (external standard), were floated on 500  $\mu$ l drops of "Buffer" for 8 min prior to washing with water and subsequent dehydration. No difference was observed if the buffer wash were omitted.

#### RESULTS AND DISCUSSION

Specificity of anti-pm<sup>7</sup>G - The binding of the purified anti-pm<sup>7</sup>G to pm<sup>7</sup>G and to GMP was determined by radioimmunoassay. The results (Fig. 1) show that binding to pm<sup>7</sup>G was with an affinity about 2000X greater than binding to GMP. AMP was even less reactive. Binding to UMP and CMP were insignificant. Since there are about 2000 GMP residues to each pm<sup>7</sup>G residue in an ASV molecule, we can expect antibody to bind, on the average, one to two randomly

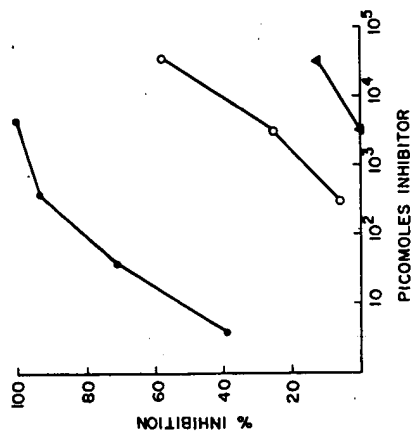


Fig. 1. Inhibition studies on anti-pm<sup>7</sup>G. ●, pm<sup>7</sup>G; ○, GMP; ▲, AMP.

located places on the RNA strand in addition to the binding to the pm<sup>7</sup>G cap at the 5'-end, perhaps where several GMP residues can react with the same antibody molecule and give cooperative binding. It was with this prediction in mind that we carried out and analyzed the electron microscopy studies. Analysis of the reaction of ASV with anti-pm<sup>7</sup>G - ASV-RNA, even as the 35S subunit, has considerable secondary structure, disruptable by treatment with gene 32 protein<sup>17</sup>. Since preliminary experiments (to be pursued further) had indicated that the pm<sup>7</sup>G in the cap of native ASV-RNA might not be available for reaction with antibody and because we did not know the effect of secondary structure on the availability of other nucleotides, we sought at this time to develop procedures in which the RNA could be denatured before reaction with antibody. Conditions that denature secondary structure also will prevent antibody-RNA binding interactions. We chose, therefore, to apply the RNA to the grid under denaturing conditions and then to expose the RNA on the grid to the appropriate antibody. This procedure produced background binding of antibody to the grid, but it can be minimized by choosing the correct conditions (Experimental). The RNA, although anchored to the grid at some points, seemed not to be immobilized completely. Strand shifting and elongation appeared to be possible even while

the RNA was attached to the grid (see below).

Examination of the grids prepared in this way showed, as reported by others<sup>18,19</sup> that ASV-RNA is heterogeneous in length. Only the longest strands were selected for photography and analysis. Our selection was otherwise impartial since any long, unambiguously spread molecule in a field was photographed. A histogram (Fig. 2) of 106 molecules shows a broad distribution with 89 molecules averaging  $1.92 \pm 0.13 \mu\text{m}$  and 17 molecules averaging  $2.39 \pm 0.14 \mu\text{m}$ . The former is consistent with  $2.1 \mu\text{m}$  for the deleted<sup>19</sup> and the latter with  $2.86 \mu\text{m}$ <sup>20</sup> for the non-deleted species. This agrees with about 16% non-deleted molecules in our preparation which, according to Guntaka (personal communication), who provided the sample, is to be expected. For the deleted species, Junghans *et al.*<sup>21</sup> reported a value of 79.7% of the intact genome; ours calculates as 80.3%, a good agreement. If we accept the value of  $2.5 \mu\text{m}$  for the distance between neighboring bases<sup>22</sup>, the non-deleted RNA calculates as 9.56 kb for the undeleted virus<sup>19,23</sup>. Using  $\phi\text{X-174}$  as an external standard, we obtained values of 10.3 and 8.31 kb for the non-deleted and deleted forms, respectively.

The positions of anti-pm<sup>7</sup>G on 70 RNA molecules having an antibody at one end are shown in Fig. 3A and B. In Fig. 3C are 16 undeleted molecules

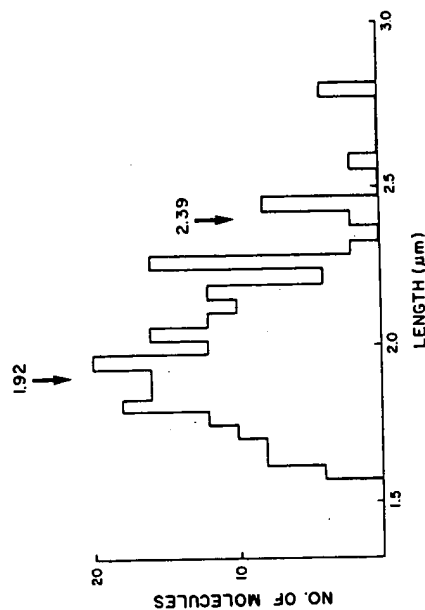


Fig. 2. Histogram showing distribution of lengths of 106 molecules of RNA.

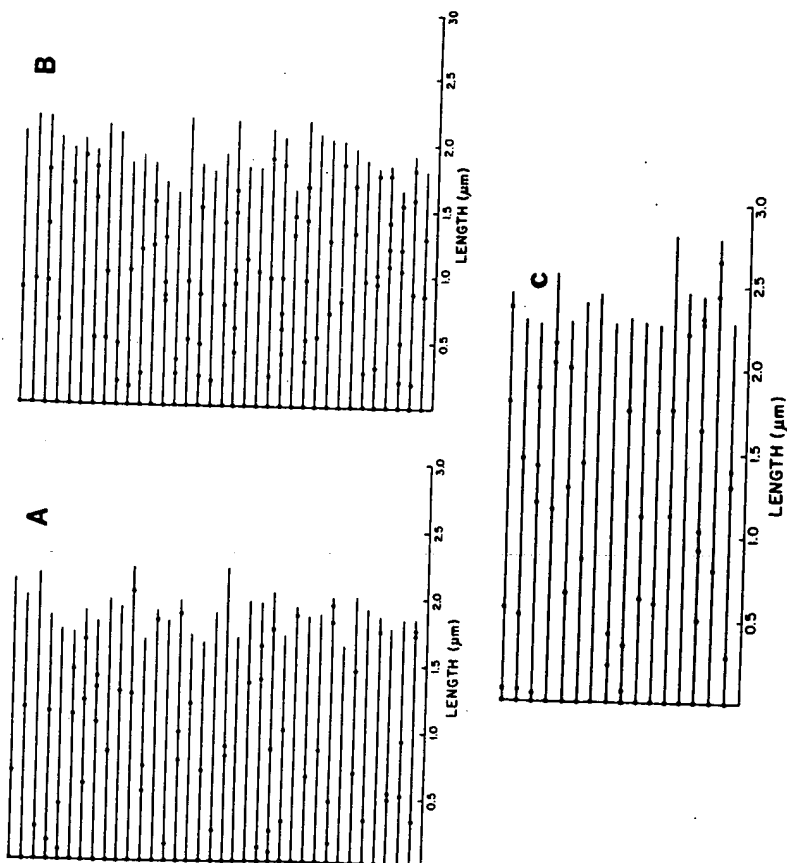


Fig. 3A, B. Positions of anti-pm<sup>7</sup>G molecules on 70 deleted RNA molecules having an antibody at one terminus. C. Positions of anti-pm<sup>7</sup>G molecules on 16 undeleted RNA molecules with an antibody at one terminus.

with antibody at one end. Four molecules were seen with antibody at both ends and 16 without antibody at termini (data not shown). Of the 106, 81% had antibody at one end, 3.8% at both ends, and 15.1% had none on the ends.

Fig. 4 is a histogram of antibody distribution along 86 of the molecules with antibody at one end. All antibody molecules located within 0.09  $\mu$ m of each other were taken as being at one location. The other RNA molecules were not used in the histogram because, by inspection, it was obvious they would not affect the histogram substantially.

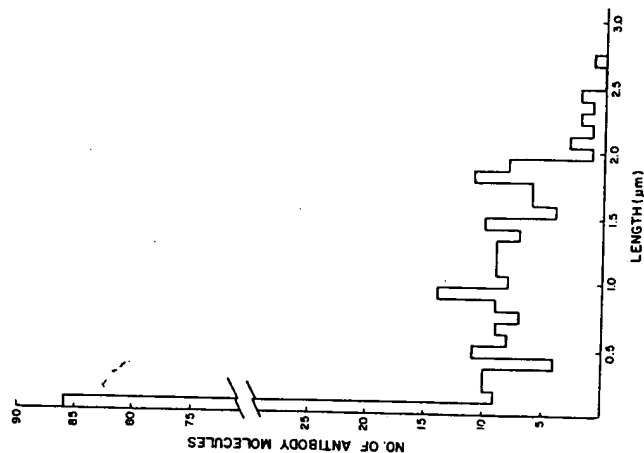


Fig. 4. Histogram of data derived from Figs. 3A, B and C, i.e. distribution of anti-pm<sup>7</sup>G molecules along strands of RNA.

The 106 molecules averaged  $3.15 \pm 0.99$  molecules of antibody per molecules of RNA, including the molecule at the terminus. ASV is 26.7% guanine<sup>24</sup>. Assuming the deleted RNA has the same percentage of guanine, there is good agreement with the relative specificities of anti-pm<sup>7</sup>G for pm<sup>7</sup>G and GMP (Fig. 1). The histogram (Fig. 4) also shows a completely random arrangement of the antibodies that bound G residues within the RNA strand.

Shown in Fig. 5 is an example of a field spread in the absence of antibody.

Shown in Fig. 6 are some examples of fields seen in the electron microscopy of preparations of RNA treated with anti-pm<sup>7</sup>G. The strands are extended to varying degrees, i.e. they have different amounts of remaining secondary structure. The strand on the upper right is highly extended; the one on the upper left still has considerable secondary structure, which can be well visualized



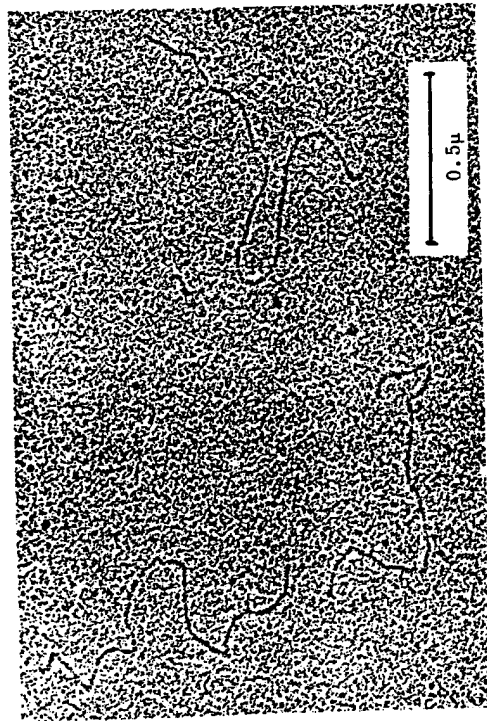


Fig. 5. ASV-RNA spread in the absence of antibody. Molecule on lower left is a fragment.

at the magnification used to measure the length of the RNA molecule (i.e. about 113,000X). All have antibody molecules at one terminus and up to three molecules bound internally.

Less than 1% of the ASV-RNA controls (i.e. RNA untreated with antibody) showed particles or secondary structure that could be mistaken for antibody and even fewer ends that were subject to question (Fig. 5).

Grids of ASV reacted with anti-pm  $^7\text{G}$ , but inhibited by pm  $^7\text{G}$ , contained very few molecules (less than 10%) with internal antibody and less than 5% with antibody at an end.

Studies of the reaction of ASV-RNA with other antibodies - We had shown earlier by immunochemical means<sup>25</sup> that anti-A could bind poly A. Grids reacted with anti-A showed many antibodies bound to ASV with especially heavy labelling at one end, presumably the poly A tail, which also appeared "kinked" as a result of reaction with antibody (Fig. 7). The distribution of the antibodies bound to the internal portions of the RNA was uneven. The RNA strands were highly extended as a result of multiple site reactions with

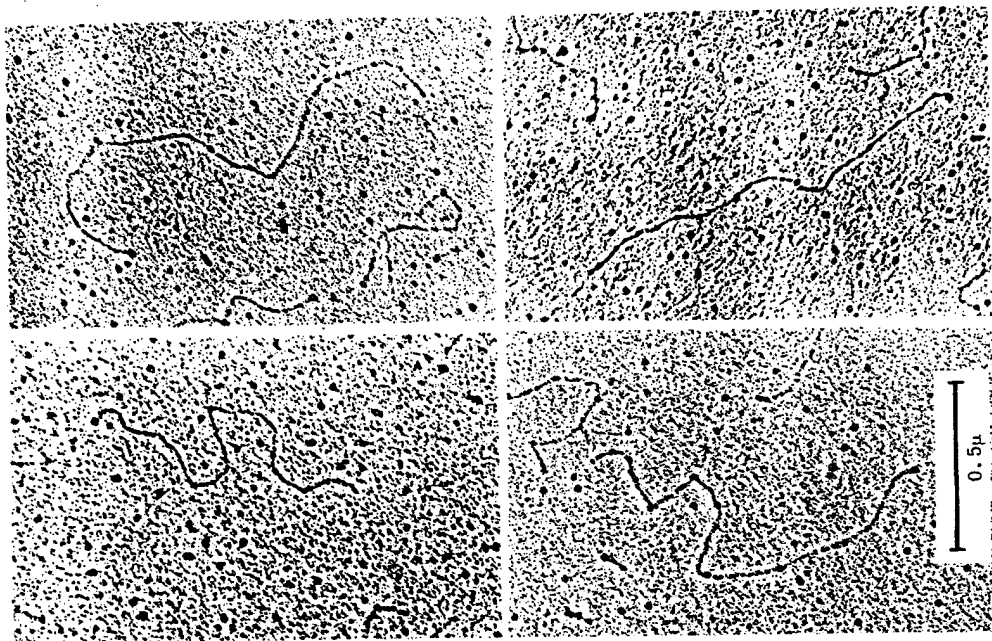


Fig. 6. Examples of ASV-RNA strands with terminal anti-pm  $^7\text{G}$  molecules.

antibody, much as was seen by Delius et al.<sup>17</sup> with gene 32 protein.

Our next studies were with anti-poly A, which had been shown to bind poly A but not AMP. A<sub>10</sub> was also bound by anti-poly A but with an apparent

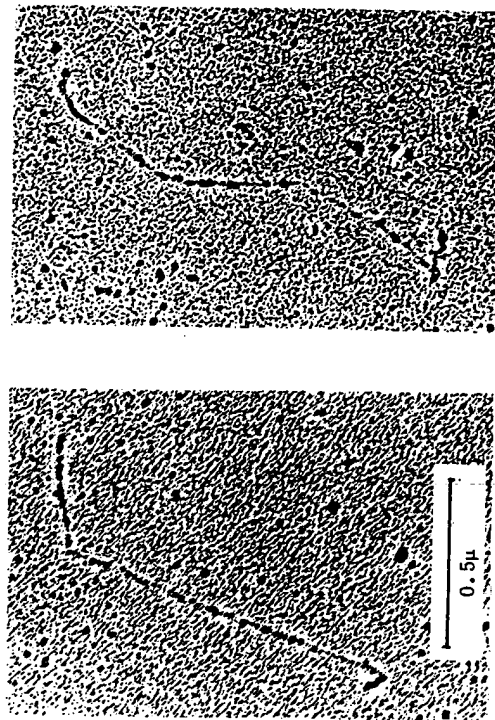


Fig. 7. Examples of ASV-RNA strands after reaction with anti-A.

binding constant several orders of magnitude less than that of poly A. In other words, its specificity was toward a conformation that could be assumed by  $(A)_n$  where  $n \geq 10$ . Grids from reaction with anti-poly A showed 1-3 antibody molecules at one end, presumably the poly A tail and from 1-4 internal antibody molecules which appeared to be at specific sites, although a statistical study will be necessary (Fig. 8). Wang and Duesberg<sup>26</sup> reported preliminary evidence for A-rich runs in addition to the 3' poly A tail of 200 nucleotides, based on observed excessive binding of 11S fragments of Rous Sarcoma Virus to oligo-(dT)-cellulose. We plan to map the anti-poly A binding sites as well as the heavier binding regions of anti-A to identify poly A and/or A-rich regions of ASV, in conjunction with studies on  $\phi$ X-174, whose sequence is known<sup>16</sup>.

Anti-C reacted with ASV-RNA, binding throughout the molecule (data not shown). Fields showed some molecules with what could be interpreted as having an unreacted poly A "tail," equivalent in size to about 300 residues. It is known (Schwartz and Gilbert, unpublished, cited in ref. 23) that C residues exist in the STR portion of the genome just preceding the poly A tail

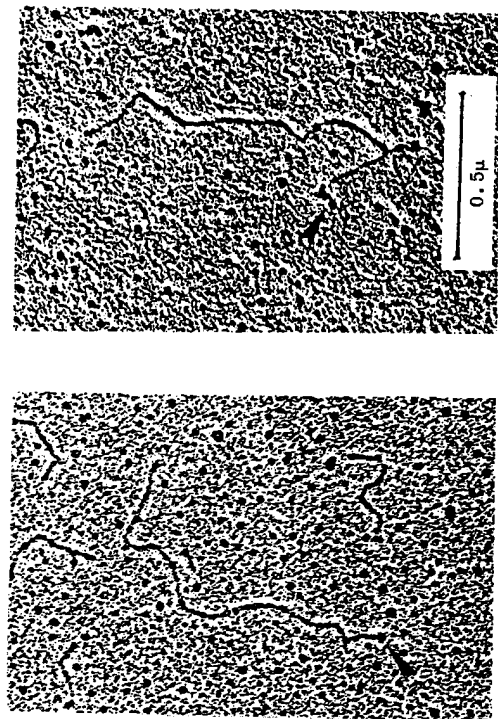


Fig. 8. Examples of ASV-RNA strands after reaction with anti-poly A. One has a single antibody molecule at one end of the RNA. The other has several. Arrowheads point to antibody.

with its approximately 200 residues. The unreacted end of the molecule seen with anti-C is somewhat longer than would be expected if the C residues in STR reacted with antibody. However, as yet, we have not examined a sufficient number of molecules. On the other hand, Junghans *et al.*<sup>21</sup> measured the poly A tail of ASV as 292 nucleotides, a discrepancy they interpreted as due to slippage of their hybrids.

One other aspect of native ASV-RNA should be mentioned: tRNA<sup>trp</sup> is bound to the RNA, 101 residues from the 5'-terminus of the genome<sup>27, 28, 29</sup>. Its interaction with the genome involves residues 2-15 as measured from its 3'-terminus<sup>30</sup>. Additional binding might also involve nucleotides 18-26<sup>27</sup>.

Although we have been able to visualize samples of tRNA with the procedures used in this work (unpublished), we have not detected any on the ASV-RNA. This is to be expected considering the denaturing conditions used to prepare the 35S subunits from the 70S molecule, as well as our conditions of spreading.

The results reported above indicate that anti-nucleotide antibodies can

be used as probes to reveal information about the location of certain purine and pyrimidine bases in single-stranded regions of nucleic acids. Our data on anti-pm<sup>7</sup>G are very convincing in this regard. More work has to be done with the other antibodies. In the procedure we used here, we chose to denature the RNA by spreading on the grid before reacting with antibody. This was done because ASV-RNA has considerable secondary structure<sup>17</sup>. Future studies will explore the reaction of antibodies with ASV-RNA before denaturation. In this way, we should get information on secondary structure and the availability of the cap in native RNA. Experiments with other nucleic acids are also in progress, as well as studies in which antibodies of other specificities are being used.

#### ACKNOWLEDGMENTS

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## Analysis of the Genome of an Endogenous, Ecotropic Retrovirus of the AKR Strain of Mice: Micromethod for Detailed Characterization of High-Molecular-Weight RNA

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A detailed characterization of the genome of an endogenous, ecotropic type C virus, the Akv virus, is presented. Approximately 100 RNase T<sub>1</sub>-resistant oligonucleotides characteristic of the Akv genome were identified by two-dimensional gel electrophoresis, and the complete nucleotide sequence is presented for 75 of these oligonucleotides. A correspondence between the sequence of some of these oligonucleotides and the amino acid sequence of some virus-coded *gag* gene proteins is reported. For this study we developed methods suitable for the analysis of high-molecular-weight RNA species in nanogram quantities. The *in vitro* labeling procedures used here led to uniform labeling of the unique oligonucleotides.

The high incidence of spontaneous leukemia in several inbred strains of mice is associated with the persistent expression of endogenous retroviruses (30, 35). Studies of genetic factors determining retrovirus expression in the AKR strain of mice have identified two dominant genetic loci (*Akv-1* and *Akv-2*) coding for the information for complete virus genomes (6). The viruses produced by these two loci are identical or very closely related (29). The relationship of this virus, here termed the Akv virus, to the ultimate appearance of thymic lymphomas remains unclear. The virus is persistently expressed in the animals from early in life, but the thymic leukemia does not develop until after 6 months of age. Although the virus itself is not oncogenic, its expression is correlated with the induction of the disease, since crosses of AKR mice with strains that restrict the expression of Akv virus do not develop spontaneous thymic leukemia (21, 30).

The recent isolation of several other groups of viruses with distinct biological properties from this strain of mice (17, 18, 22, 33) has prompted speculations regarding the role of virus variants in the leukemogenic process. To elucidate the mechanisms for the generation of this diverse family of viruses in AKR mice and to understand this variation in relation to the development of the disease, we have begun a detailed characterization of the genomes of viruses of this family.

As sensitive tools for a rapid characterization of high-molecular-weight RNA, we used the techniques of oligonucleotide mapping and RNA sequence analysis. To enable the characterization of RNA of viruses produced in low titers in tissue culture, as well as viruses from animal

tissues, we adapted these techniques to the analysis of nonradioactive RNA in nanogram quantities.

Here we describe techniques for the isolation of retrovirus RNA in small amounts, as well as procedures for the analysis of complex RNA in femtomole quantities. We have found this micromethod to be generally useful in the study of genomes of a large number of retroviruses, as well as other RNA viruses. It involves specific RNase T<sub>1</sub> digestion of RNA in small quantities, followed by uniform labeling of the RNase-resistant oligonucleotides at the 5' termini by <sup>32</sup>P at a high specific activity. The radioactive oligonucleotides are separated by a high-resolution two-dimensional gel electrophoresis system, and the oligonucleotide pattern characteristic of that particular RNA sample is visualized by autoradiography. The unique oligonucleotides are further characterized by recently developed techniques for rapid nucleotide sequence determination of RNA labeled at a terminal position (10, 16, 32).

In this paper we apply these techniques in a detailed analysis of the genome of the Akv virus. The complete nucleotide sequences of 75 large RNase T<sub>1</sub>-resistant oligonucleotides accounting for a total of 10 to 15% of the genome have been determined. The results of the nucleotide sequence analysis are discussed and correlated with the available amino acid sequence information for the viral structural proteins.

### MATERIALS AND METHODS

**Virus isolates.** Akv virus produced by an AKR embryo fibroblast cell line cloned for spontaneous virus production was provided by Electro-Nucleonics

Laboratories, Inc. (lot no. 5012-29-58 and 5012-47-59). Akv virus, chemically induced from an AKR embryo fibroblast line and infected onto NIH 3T3 mouse embryo fibroblasts, was provided by N. Copeland (7). The virus-producing cells were grown in Dulbecco-modified Eagle medium containing 5 to 10% bovine serum.

**Enzymes.** RNase T<sub>1</sub> was purchased from Calbiochem (Sankyo). The enzyme was dissolved in 20 mM Tris-hydrochloride (pH 8.0)-2 mM EDTA and stored at -20°C. The enzymatic activity was determined as described previously (25). The number of units of enzyme activity determined usually corresponded to approximately two times the manufacturer's specifications. Bacterial alkaline phosphatase was purchased from Boehringer Mannheim Corp. as a suspension in 3.2 M ammonium sulfate. The precipitate was pelleted by centrifugation and dissolved in 0.03 M sodium cacodylate (pH 8.0), and the ammonium sulfate was removed by passage through a Sephadex G-25 column. The enzyme solution was stirred for 1 h at 0°C with 0.1% diethylpyrocarbonate (Eastman Kodak Co.). The diethylpyrocarbonate was removed by extraction with ether, and the enzyme solution was stored at -20°C. The phosphatase activity was determined spectrophotometrically as the rate of release of *p*-nitrophenol from a 0.001 M solution of *p*-nitrophenylphosphate in 1.0 M Tris-hydrochloride (pH 8.0). One unit is the activity liberating 1 μmol of *p*-nitrophenol per min at 25°C (36). The molar absorbance index for *p*-nitrophenol in 1.0 M NaCl (pH 8.0) is  $1.62 \times 10^4$ .

Polynucleotide kinase from T4-infected *Escherichia coli* was supplied by P. L. Biochemicals. RNases T<sub>2</sub> and U<sub>2</sub> were purchased from Calbiochem (Sankyo), and RNase A was from bovine pancreas from Worthington Biochemicals Corp.

A preparation of a mixture of extracellular RNases from *Physarum polycephalum* (2, 26) was provided by H. Donis-Keller or prepared by us as a 20-fold concentrate of a cell- and slime-free supernatant of a late exponential culture of *P. polycephalum*. The supernatant was concentrated by lyophilization and dialyzed against 10 mM NaOAc, pH 4.5. The enzyme preparation was stored in 40% glycerol at -20°C for up to 1 year without any loss of activity.

**Preparation of [ $\gamma$ -<sup>32</sup>P]ATP.** A simple reproducible method for the synthesis of high-specific-activity [ $\gamma$ -<sup>32</sup>P]ATP was adapted from existing procedures (15, 20). [ $\gamma$ -<sup>32</sup>P]ATP at a specific activity of about 2,000 Ci/mmol was prepared by isotope exchange for 20 to 30 min at room temperature in a solution of the following composition: 100 mM Tris-hydrochloride (pH 8.0), 7 mM Mg(OAc)<sub>2</sub>, 0.5 mM EDTA, 2 mM reduced glutathione, 0.3 mM 3-phosphoglycerate, 0.4 mM ATP, 1.4 Ci of [<sup>32</sup>P]phosphate (New England Nuclear Corp.; carrier-free [<sup>32</sup>P]orthophosphoric acid in 0.02 M HCl, lyophilized before use) per ml, 100 mg of 3-phosphoglycerate kinase per ml, and 500 mg of glyceraldehyde-3-phosphate dehydrogenase per ml. The enzymes were purchased from Boehringer Mannheim Corp. as a mixed suspension at 6 mg/ml in 3.2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Immediately before use, the precipitate was pelleted by centrifugation from a 50-μl sample of the enzyme suspension, dissolved in 50 μl of 10 mM Tris-hydrochloride (pH 8.0)-2 mM reduced glutathione-0.1 mM

$\beta$ -nicotinamide adenine dinucleotide (Sigma), and dialyzed against a solution of the same composition at 0°C for 15 to 30 min. The incorporation of <sup>32</sup>P into ATP was monitored by ascending thin-layer chromatography on polyethyleneimine cellulose sheets (Brinkmann Instruments Inc.) in 0.75 M KH<sub>2</sub>PO<sub>4</sub> (pH 3.5), followed by autoradiography. When more than 50% of the radioactive isotope was incorporated into ATP, the reaction was terminated by the addition of 3 volumes of 7 mM EDTA, followed by extraction with an equal volume of phenol (redistilled and saturated with 10 mM Tris-hydrochloride, pH 8.0). The phenol was removed from the aqueous phase by ether extraction, and water was added to give a final ATP concentration of 25 μM. The [ $\gamma$ -<sup>32</sup>P]ATP solution was stored in aliquots at -20°C and used in the polynucleotide kinase reaction without any further treatment.

**Isolation of viral RNA.** Concentrated virus pellets were suspended into 1 ml to a few milliliters of 50 mM Tris-hydrochloride (pH 7.5)-100 mM NaCl-1 mM EDTA and purified by sedimentation through 0.88 M sucrose (30%, wt/vol) in the same solution for 2 h at 50,000 rpm in an SW50 rotor. The virus pellets were disrupted in a solution of 10 mM Tris-hydrochloride (pH 7.4), 450 mM NaCl, 45 mM sodium citrate, 1 mM EDTA, 10 g of sodium dodecyl sulfate per liter, and 0.2 mg of proteinase K (E. Merck AG, predigested for 30 min at 37°C) per ml by incubation for 2 h at 37°C.

The 70S RNA was purified by centrifugation through a linear gradient of 0.44 to 0.88 M sucrose in 50 mM Tris-hydrochloride (pH 7.5)-100 mM NaCl-1 mM EDTA-2 g of sodium dodecyl sulfate per liter in an SW41 rotor at 38,000 rpm for 120 min at 25°C. Gradient fractions containing 70S RNA were located by measurement of the absorption at 260 nm, pooled, diluted with an equal volume of 600 mM NaCl-60 mM sodium citrate, and applied to a column of oligodeoxythymidylic acid [oligo(dT)]-cellulose (Collaborative Research, Inc.; T-3; column size, 3-mm inside diameter by 15 mm) in 10 mM Tris-hydrochloride (pH 7.5)-300 mM NaCl-30 mM sodium citrate. The column was washed with 5 ml of a solution of the same composition, and the RNA was eluted in 0.4 ml of 10 mM Tris-hydrochloride (pH 7.4) into a siliconized polypropylene microtest tube (Eppendorf). A 40-μl amount of 3 M NaOAc-(pH 6.0)-0.1 M Mg(OAc)<sub>2</sub>-0.01 M EDTA and then 1.1 ml of 95% EtOH were added, and the tube was chilled for 20 min at -70°C. The precipitated RNA was pelleted by centrifugation for 20 min at about 10,000 × *g* in a microcentrifuge, dissolved in 0.4 ml of water, and reprecipitated under the same conditions. The RNA pellet was washed with 200 μl of 95% ethanol, dried, dissolved in 5 to 10 μl of 20 mM Tris-hydrochloride (pH 8.0)-2 mM EDTA, and stored at -20°C. The amount of RNA was determined from a UV absorption spectrum of an RNA solution, using the difference  $A_{260} - A_{300} = 1.0$  for a solution containing 50 μg of RNA per ml in a cuvette of a 10-mm path length, where  $A_{260}$  and  $A_{300}$  represent the absorbances at 260 and 300 nm, respectively.

For the isolation of 35S RNA, 70S RNA at a concentration of about 100 μg/ml in 5 mM EDTA (pH 7.0) was denatured by heating for 5 min at 95°C. Full-length polyadenylic acid-containing viral RNA was isolated by chromatography of the denatured RNA

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**RNAse T<sub>1</sub> digestion of viral RNA.** Viral RNA at a concentration of between 1 and 250 ng/ $\mu\text{l}$  was digested with RNAse T<sub>1</sub> for 60 min at  $37^\circ\text{C}$  in siliconized polypropylene microtest tubes (Eppendorf) in a solution of the following composition: 0.5 U of RNAse T<sub>1</sub> per  $\mu\text{l}$  and  $2.5 \times 10^{-4}$  U of bacterial alkaline phosphatase per  $\mu\text{l}$  in 20 mM Tris-hydrochloride (pH 8.0)-2 mM EDTA.

**$^{32}\text{P}$  labeling of the oligonucleotides.** The RNAse T<sub>1</sub>-resistant oligonucleotides were labeled with  $^{32}\text{P}$  by the addition of 3 to 100  $\mu\text{l}$  of the polynucleotide kinase reaction mixture directly to the RNAse digestion mixture. The polynucleotide kinase reaction mixture had the following composition: 10 mM potassium phosphate (pH 9.5), 10 mM  $\text{Mg}(\text{OAc})_2$ , 5 mM dithiothreitol, 15  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP, and 40 U of polynucleotide kinase per ml. The tube was incubated at  $37^\circ\text{C}$  for 4 h. The labeling reaction was terminated by the addition of an equal volume of 0.6 M  $\text{NH}_4\text{OAc}$  with 2 mg of yeast carrier RNA per ml. This mixture was then extracted with an equal volume of phenol (distilled and saturated with 10 mM Tris-hydrochloride, pH 8.0, at room temperature). The RNA was precipitated from the aqueous phase with 3 volumes of ethanol at  $-70^\circ\text{C}$  for 20 min, pelleted by centrifugation, lyophilized, and dissolved in 5 to 15  $\mu\text{l}$  of the gel loading mixture (1 mM EDTA [pH 7.0], 7 M urea, 0.1% bromophenol blue, 0.1% xylene cyanol FF, and 200  $\mu\text{g}$  of yeast carrier RNA per ml). A sample of the solution was layered onto the first-dimension gel.

**Two-dimensional gel electrophoresis.** Two-dimensional gel electrophoresis was carried out by using a modification of the system described by deWachter and Fiers (9). The first-dimension gel had the dimensions 40 cm by 20 cm by 0.75 mm and the following composition: 10% acrylamide, 0.3%  $N,N'$ -methylenebisacrylamide, 6 M urea, and 25 mM citric acid (pH 3.5). The gel was submerged in electrophoresis buffer (25 mM citric acid, pH 1.9), and electrophoresis was carried out at room temperature at 1,700 V for about 2 h until the front dye (bromophenol blue) had migrated 18 cm. Gel strips (1.5 cm wide) corresponding to 4 to 35 cm from the origin were cut out and placed between the glass plates for the second-dimension gel. The second-dimension gel had the dimensions 40 cm by 33 cm by 0.38 mm. The dimension of the second gel was deliberately reduced to insure proper mounting of the gel strip. The composition of the second-dimension gel was as follows: 22.8% acrylamide, 0.8%  $N,N'$ -methylenebisacrylamide, and TEB (50 mM Tris-borate [pH 8.3], 1 mM EDTA). The gel mixture was poured around the strip from the first-dimension gel, which was located parallel to the bottom of the mold at a 4-cm distance. Electrophoresis was carried out at room temperature in TEB at 500 to 1,500 V until the bromophenol-blue marker had migrated 25 cm. Auto-

radiography of the gel was carried out with Kodak XR-2 film at  $4^\circ\text{C}$  or at  $-70^\circ\text{C}$  with intensifying screens.

**Nucleotide sequence analysis.** Gel pieces containing the 5'-labeled oligonucleotides were cut out of the second-dimension gels, and the RNA was eluted by diffusion for 2 h at  $37^\circ\text{C}$  into 200  $\mu\text{l}$  of a solution containing 1 mM EDTA (pH 7.0) and 200  $\mu\text{g}$  of yeast carrier RNA per ml. The solution was made 0.3 M  $\text{NaOAc}$ , 0.01 M  $\text{Mg}(\text{OAc})_2$ -0.1 mM EDTA (pH 6.0), and the RNA was precipitated with 3 volumes of ethanol for 20 min at  $-70^\circ\text{C}$ . The precipitated RNA was pelleted by centrifugation, and the pellets were washed with 200  $\mu\text{l}$  of 95% ethanol, lyophilized, and dissolved in 10  $\mu\text{l}$  of 20 mM Tris-hydrochloride-2 mM EDTA (pH 7.5).

For nucleotide sequence analysis, a sample of the solution of the 5'-labeled oligonucleotide containing between 5,000 and 100,000 dpm was added to 35  $\mu\text{l}$  of the reaction mixture (10 of 20 mM sodium citrate, 1 mM EDTA, 7 M urea, 0.25  $\mu\text{g}$  of yeast carrier RNA per ml, and 0.025% (wt/vol) of xylene cyanol FF and bromophenol blue, adjusted with citric acid to pH 5.8. Seven 5- $\mu\text{l}$  aliquots were added to the wells of a microtiter plate (containing  $8 \times 12$  conical wells; Cooke Laboratory Products; MC2000). The following additions were made to the aliquots: (no. 1) no addition (control); (nos. 2 and 3) 1  $\mu\text{l}$  of RNAse A in 20 mM Tris-hydrochloride (pH 7.5)-2 mM EDTA at 2.5 and 0.125 ng/ $\mu\text{l}$ , respectively; (nos. 4 and 5) 1  $\mu\text{l}$  of RNAse U<sub>2</sub> in the reaction mixture at 0.25 and 0.025 U/ $\mu\text{l}$ , respectively; (no. 6) 1  $\mu\text{l}$  of an appropriate amount of *Physarum* RNases in 10 mM sodium citrate (pH 5.0)-50% glycerol; (no. 7) 1  $\mu\text{l}$  of RNAse T<sub>2</sub> at 0.005 U/ $\mu\text{l}$  in the reaction mixture. The microtiter plate with the aliquots was incubated in a water bath for 1 h at  $50^\circ\text{C}$ . At this point, the samples were either layered immediately onto the gel or stored at  $-20^\circ\text{C}$  before loading.

For mapping of RNAse cleavage sites, we used polyacrylamide gels of the dimensions 40 cm by 31 cm by 0.75 mm and the following composition (10): 24% (wt/vol) acrylamide, 0.7% (wt/vol)  $N,N'$ -methylenebisacrylamide, 7 M urea, 50 mM Tris-borate (pH 8.3), 1 mM EDTA, 0.7 g of ammonium persulfate per liter, and 100  $\mu\text{l}$  of  $N,N,N',N'$ -tetramethylethylenediamine. Each gel contained 30 wells for layering of samples. The gel was subjected to pre-electrophoresis for 2 h at 1,000 V before loading. Electrophoresis was for 5 to 7 h at 1,300 V until the bromophenol blue marker had migrated 19 cm from the origin. For autoradiography of the gels, we used a Kodak regular intensifying screen and Kodak XR film.

For the determination of 5'-nucleotides, an aliquot of the solution of the 5'-labeled oligonucleotides containing 1,000 to 10,000 dpm was added to 30  $\mu\text{l}$  of 1.0 M piperidine, and the RNA was hydrolyzed by incubation for 10 to 20 h at  $50^\circ\text{C}$ . Aliquots (5  $\mu\text{l}$  each) of the hydrolyzed samples were applied to polyethyleneimine cellulose thin-layer chromatography plates (Brinkmann Instruments Inc.). The plates were developed in 0.5 M  $\text{KH}_2\text{PO}_4$ , pH 3.5. The radioactive nucleotides were localized by autoradiography with a Kodak regular intensifying screen and Kodak XR film. As markers for the identification, we used 5',3'-nucleoside diphosphates carrying [ $^{32}\text{P}$ ]phosphate groups in



the 5' position. These were synthesized from 3'(2')-AMP, 3'(2')-UMP, 3'(2')-CMP, and 3'(2')-GMP (Sigma Chemical Co.) by the T4 polynucleotide kinase-catalyzed transfer of [ $^{32}$ P]phosphate from [ $\gamma$ - $^{32}$ P]ATP to 3'-nucleoside monophosphates. These reactions were carried out in the polynucleotide kinase reaction mixture as described previously in this paper for the oligonucleotide labeling. The concentrations of 3'(2')-nucleoside monophosphates in the labeling reaction were 15  $\mu$ M. The reactions were terminated and phenol extracted as described for the oligonucleotide labeling. The phenol was removed by ether extraction, and the markers were stored frozen for up to several months at  $-20^{\circ}\text{C}$ .

**Analysis of homogeneously labeled RNA.**  $^{32}$ P-labeled Akv virus was prepared from supernatant medium isolated from a culture labeled with  $^{32}\text{PO}_4$  in low-phosphate medium (1).  $^{32}$ P-labeled 70S RNA was isolated as described above and digested by RNase T<sub>1</sub> at 50 U/100  $\mu$ g of carrier RNA in a 10- $\mu$ l volume for 30 min at  $37^{\circ}\text{C}$  (1).

## RESULTS

Two Akv viruses were used for this study. One was derived by induction with iododeoxyuridine of a nonproducing AKR embryo fibroblast cell line. This virus, here called Akv(I), was grown on a line of NIH 3T3 mouse embryo fibroblasts. Virus produced by a line of AKR fibroblasts that had been cloned and that spontaneously produced virus was also used [Akv(S)]. Previous work with these cell lines showed that the virus produced was ecotropic, N tropic, and fusion positive (XC<sup>+</sup>) (7, 22).

**Isolation of 70S RNA.** We wished to develop procedures suitable for the isolation of RNA from cultures that produced very low titers of virus. The details of the procedure are given above and are similar to previously published methods (1, 12). The modifications of the standard protocols described permit the isolation of 70S RNA in the 10- to 50-ng range, starting with several liters of tissue culture medium containing virus in very low titers.

**Analysis of RNA.** The methods used in the analysis of the RNA include the following steps. The RNA is digested to completion with RNase T<sub>1</sub>. The products of this digestion are oligonucleotides carrying a single guanosine residue, which is located at the 3' end. The external phosphate group carried by this residue is subsequently removed by a bacterial alkaline phosphatase. The oligonucleotides are then radioactively labeled by [ $^{32}$ P]phosphate. For this labeling, we use the T4 polynucleotide kinase (27)-catalyzed transfer of radioactive phosphate from [ $\gamma$ - $^{32}$ P]ATP to the 5'-hydroxyl groups of the 5'-terminal residues of the oligonucleotides. The RNase T<sub>1</sub>-resistant oligonucleotides are then separated by two-dimensional gel electrophore-

sis, and the patterns of RNase T<sub>1</sub>-resistant oligonucleotides (termed an RNase T<sub>1</sub> fingerprint) are detected by autoradiography. For further analysis, the 5'-labeled RNase T<sub>1</sub>-resistant oligonucleotide can be eluted from the gel and the nucleotide sequence can be determined.

The successful use of these techniques depends upon reliable procedures for the following: (i) specific RNase T<sub>1</sub> digestion of high-molecular-weight RNA in femtomole quantities; (ii) uniform radioactive labeling of RNase T<sub>1</sub>-resistant oligonucleotides; (iii) labeling of oligonucleotides at a high specific activity to allow nucleotide sequence analysis; (iv) two-dimensional gel electrophoresis that permits the detection and isolation of a maximum number of unique oligonucleotides; (v) routine nucleotide sequence analysis of a large number of 5'-labeled oligonucleotides.

Although previous methods for this kind of analysis (13, 34) exist, we found that none of these would satisfy all of the above conditions. We therefore undertook the task of redefining the conditions for the enzymatic reactions involved, as well as improving the conditions for the fractionation of oligonucleotides. Below we shall present, step by step, the details of the experimental conditions that we decided upon and subsequently illustrate their use in the analysis of the genome of Akv virus.

**RNase digestion and phosphatase treatment.** During the studies of conditions for the specific RNase T<sub>1</sub> digestion of RNA in small quantities, we found that the cleanest digestion patterns were obtained when we used much higher enzyme-to-RNA ratios than were specified in the literature for the digestion of RNA in microgram quantities (1). As illustrated below, we found that an RNase T<sub>1</sub> concentration of 0.5 U/ $\mu$ l leads to the specific digestion of high-molecular-weight RNA in concentrations ranging from 1 to 250 ng/ $\mu$ l under the conditions specified above. The bacterial alkaline phosphatase is included in the RNase digestion mixture to insure the complete removal of the external phosphate group of the 3'-terminal residues, as wild-type T4 polynucleotide kinase contains a 3'-phosphatase activity which would lead to a partial or complete removal of the 3'-phosphate during the labeling reaction (4, 5). If the removal of the 3'-terminal phosphate is incomplete, each oligonucleotide appears as a double spot. Such doublets were observed in reactions lacking bacterial alkaline phosphatase. To inhibit the phosphatase, we include 10 mM phosphate in the polynucleotide kinase reaction mixture. Phosphate at this concentration will completely inhibit the further activity of the phosphatase, but

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uniques determined following: (i) high-molecular-weight oligonucleotides; (ii) use of  $T_1$ -resistant oligonucleotides; (iii) low molecular weight nucleotides; (iv) two-dimensional gel electrophoresis and detection and unique oligonucleotide sequence labeled oligonucleotides.

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cause only a partial reduction of the polynucleotide kinase activity (11).

As the efficient labeling of oligonucleotides derived from RNA in these quantities requires [ $\gamma$ - $^{32}$ P]ATP in the millicurie range, we prefer to use a crude preparation of [ $\gamma$ - $^{32}$ P]ATP made by enzyme-catalyzed isotope exchange. The procedure for [ $\gamma$ - $^{32}$ P]ATP synthesis described above is adapted from existing procedures to give a solution of [ $\gamma$ - $^{32}$ P]ATP at a high specific activity, free of components which might interfere with the labeling of the oligonucleotides. The exchange-reaction enzyme mixture, which is stored as a precipitate in ammonium sulfate, is dialyzed immediately before use to remove ammonium ions which are inhibitory for polynucleotide kinase (27). A phenol extraction is included after completion of the exchange reaction to remove the enzymes. We have found that this procedure reproducibly leads to the conversion of 50 to 75% of the radioactivity into ATP, giving a specific activity of approximately 2,000 Ci/mmol. When freshly made [ $\gamma$ - $^{32}$ P]ATP of this specific activity is used for the labeling reaction, the amount of radioactivity per oligonucleotide is found to be approximately 500 dpm per ng of starting material if RNA of a nonredundant sequence of about 10,000 nucleotides is analyzed. To terminate the labeling of the oligonucleotides, we used standard procedures (20). To inhibit the polynucleotide kinase, we added 0.3 M ammonium acetate together with yeast carrier RNA. The solution was deproteinized by phenol extraction, and the RNA was precipitated by ethanol. We found that the conditions used here for ethanol precipitation led to the efficient precipitation of oligonucleotides of all size classes that were of interest for the oligonucleotide mapping studies.

**Two-dimensional gel electrophoresis.** We wished to improve the resolution of the two-dimensional gel system developed by deWachter and Fiers (9) so that most of the unique RNase  $T_1$ -resistant oligonucleotides could be obtained of sufficient purity to permit sequence determination. We also wished to simplify the procedure by using our standard electrophoresis equipment for nucleotide sequence analysis at room temperature. Briefly, the modifications of the conditions of deWachter and Fiers include the use of gels of reduced thickness and increased electrophoretic dimensions to provide better separation of oligonucleotides, increased photographic resolution, and shorter electrophoresis time. For convenience, no urea is included in the electrophoresis buffer in the first dimension. The use of a Tris-borate buffer instead of a Tris-citrate buffer provides better resolution of oligonucleotides of 5 to 15 nucleotides in length, as

has been observed by others (14).

The use of this high-resolution two-dimensional gel electrophoresis system makes it possible to detect and isolate large numbers of unique oligonucleotides, including the longer oligonucleotides and medium-length oligonucleotides of unusual nucleotide composition. A majority of these oligonucleotides are pure enough for nucleotide sequence analysis by the procedures described here. This method resolves more than twice the number of unique oligonucleotides resolved by the two-dimensional gel system of deWachter and Fiers (see below).

The inclusion of 7 M urea into the second-dimension gel mixture might be preferred in some cases. A gel of this composition will give a better resolution of the shortest oligonucleotides, and the high concentration of urea will reduce the diffusion of oligonucleotides that occurs upon prolonged storage. The urea-containing gels can be frozen and thawed without breakage, a feature that is convenient for low-temperature autoradiography using intensifying screens.

**RNase  $T_1$  fingerprints of Akv virus.** Figure 1a shows an RNase  $T_1$  fingerprint of Akv RNA obtained by the techniques described above. For this experiment, 200 ng of 70S RNA was incubated with RNase  $T_1$  and bacterial alkaline phosphatase in a 2- $\mu$ l volume, followed by labeling of the oligonucleotides for 4 h in 50  $\mu$ l of the polynucleotide kinase reaction mixture. A schematic diagram of this fingerprint is shown in Fig. 1b. Approximately 100 oligonucleotides were assigned a number. These included oligonucleotides which were well resolved under these electrophoresis conditions, as well as oligonucleotides which were partially or completely overlapping with other oligonucleotides. Assuming that all unique oligonucleotides are present in equimolar amounts, uniform labeling of all 5' ends would lead to an equal amount of radioactivity in all well-resolved oligonucleotides.

To determine whether a uniform labeling efficiency was obtained, the amount of radioactivity in individual spots was determined. The amount of radioactivity in 41 oligonucleotides that were pure species (see sequencing data below) is shown in Table 1. The mean value of all determinations was calculated, and the values obtained for the individual oligonucleotides are given as a percentage relative to this number. The standard deviation was 18.9% of the mean value. It should be noted that the values obtained for the oligonucleotides of the upper right corner of the gel were among the highest due to the higher background of this part of the gel.

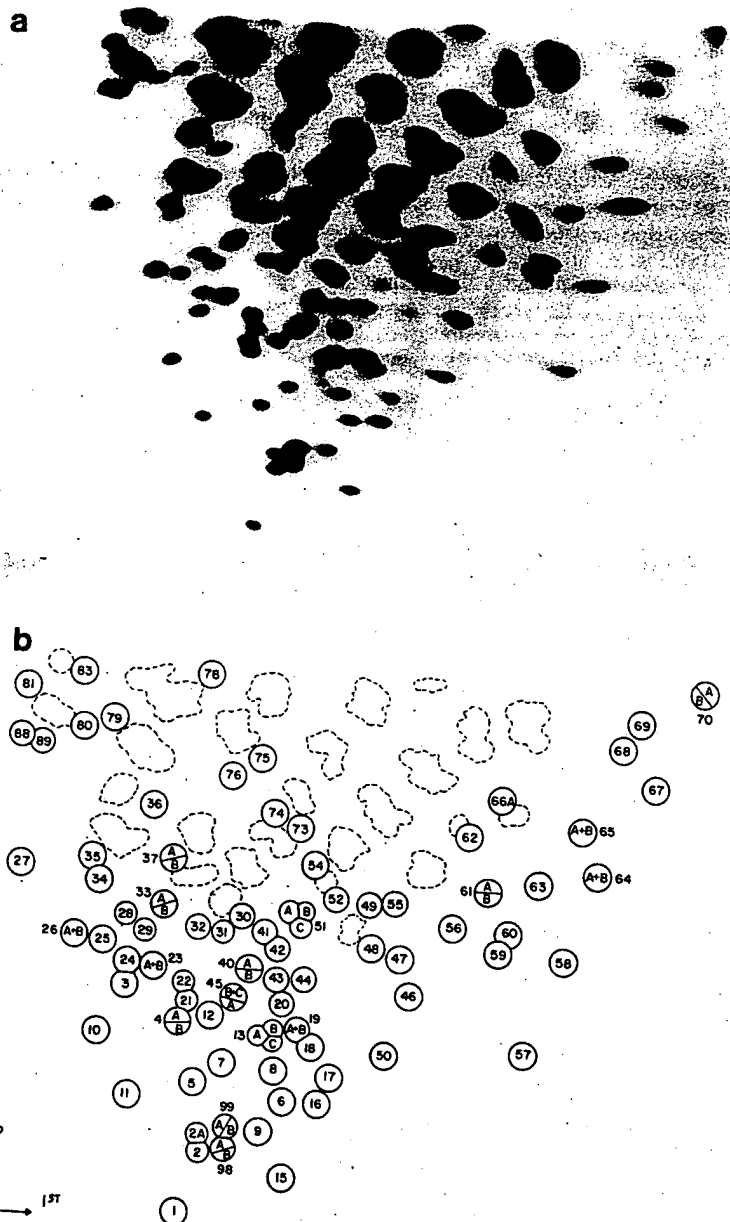


FIG. 1. (a) RNase  $T_1$  fingerprint of 70S RNA of Akv(1). A 200-ng amount of RNA was digested by RNase  $T_1$  in a 2- $\mu$ l volume, followed by 5' labeling in 50  $\mu$ l of the polynucleotide kinase reaction mixture for 4 h as described in the text. The electrophoresis directions were as follows: first dimension, from left to right; second dimension, from bottom to top. (b) Schematic diagram of the RNase  $T_1$  fingerprint of (a). The identification of the oligonucleotides is based on multiple experiments. Oligonucleotides 78, 70A, and 70B are not clearly distinguishable in this particular experiment.

Some of the oligonucleotides were clearly visible as distinct spots on the autoradiograms, but were difficult to recover completely from the gel without contamination of material from the neighboring oligonucleotides. The amount of ra-

dioactivity in gel pieces containing such groups of two or three oligonucleotides was determined (Table 1). Such oligonucleotides include, for example, no. 4A and 4B and 13A, 13B, and 13C. Furthermore, our sequence analysis (see below)

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67  
68  
69

Double

4A + 4I  
19A + 19I  
23A + 23I  
26A + 26I  
33A + 33I  
40A + 40E  
41 + 42  
61A + 61E  
64A + 64B  
65A + 65B  
98A + 98B  
99A + 99B

Triple

13A + 13B  
45A + 45B  
51A + 51B

\* Pieces collected from the gel in 10 ml of Ac amount of rac a Beckman liquid determination oligonucleotide was 18.9%

and previous that some to two olig amounts. S

TABLE 1. Determination of the amount of radioactivity in oligonucleotides of second-dimension gel<sup>a</sup>

Oligonucleotide no.	cpm $\times 10^{-3}$	% of mean value
Single		
1	8.42	75
2	9.8	88
3	9.1	82
5	9.1	82
6	9.1	82
7	9.2	82
8	11.8	106
9	10.8	96
10	9.2	83
11	8.4	75
12	12.7	114
15	9.2	83
16	11.3	101
17	9.8	88
18	10.8	97
20	12.6	114
21	12.9	116
22	9.2	83
24	9.6	86
25	10.7	96
27	10.0	90
28	8.8	79
29	9.1	82
31	14.9	134
32	11.1	100
34	9.4	85
35	10.6	95
43	12.2	110
44	11.4	103
46	12.7	114
47	12.1	109
50	11.0	99
56	14.9	134
57	ND	
58	9.8	88
59	9.8	88
60	14.7	132
63	13.8	124
67	12.2	110
68	16.9	152
69	14.6	132
Double		
4A + 4B	20.3	
19A + 19B	18.8	
23A + 23B	18.6	
26A + 26B	15.9	
33A + 33B	20.4	
40A + 40B	20.2	
41 + 42	21.7	
61A + 61B	25.7	
64A + 64B	17.8	
65A + 65B	16.7	
98A + 98B	15.4	
99A + 99B	18.6	
Triple		
13A + 13B + 13C	29.0	
45A + 45B + 45C	30.0	
51A + 51B + 51C	29.1	

<sup>a</sup> Pieces containing radioactive oligonucleotides were isolated from the gel shown in Fig. 1. The gel pieces were placed in 10 ml of Aquasol II (New England Nuclear Corp.), and the amount of radioactivity in each sample was determined with a Beckman liquid scintillation counter. The mean value of the determinations of the amount of radioactivity in the single oligonucleotides was  $11.0 \times 10^3$  cpm, and the standard deviation was 18.9% of this value. ND, Not determined.

19B and 23A and 23B. Gel pieces containing these oligonucleotides were excised from the gel, and the amount of radioactivity was determined (Table 1).

This analysis shows that the 5' labeling technique leads to a fairly uniform labeling of all unique oligonucleotides and that this method should permit the distinction between oligonucleotides present in unimolar quantities and oligonucleotides represented more than once. This degree of variation in the amount of radioactivity in individual oligonucleotides found in the present study is within the range of variations from unimolarity of single RNase T<sub>1</sub>-resistant oligonucleotides of retrovirus RNA as studied by conventional techniques (1).

To compare the RNase T<sub>1</sub> fingerprint obtained by this technique with a fingerprint derived by standard techniques, we analyzed 70S RNA of Akv virus homogeneously labeled by growth in [<sup>32</sup>P]phosphate-containing medium. The radioactive RNA was isolated by the procedure used for the isolation of nonradioactive RNA. The RNase digestion was carried out by standard procedures (1) after the addition of nonradioactive carrier RNA, and the oligonucleotide pattern was analyzed by two-dimensional electrophoresis (Fig. 2). If the RNase had the same cutting specificity under the two conditions and if all oligonucleotides carrying a free 5'-hydroxyl group were labeled in the kinase reaction, then the same oligonucleotides should be present in the pattern obtained by the two methods. The only exception might be the oligonucleotide derived from the 5' terminus of the viral RNA. If this oligonucleotide were capped, it would not contain a free 5'-hydroxyl group and should therefore show up only in the fingerprint of homogeneously labeled RNA.

When the patterns of Fig. 1 and 2 were compared, they were found to be very similar, and the identification of corresponding oligonucleotides of the two patterns was unambiguous. Moreover, fingerprints shown here are similar to those previously obtained for the homogeneously labeled [<sup>32</sup>P]RNA by Rommelaere et al. (29). The unique oligonucleotides identified by these workers are a subset of the unique oligonucleotides identified here (also see sequence data below). However, comparisons of fingerprints of uniformly labeled RNA with fingerprints of in vitro-labeled RNA show that the electrophoretic mobilities of some of the oligonucleotides are slightly different. For example, oligonucleotide 4A is reproducibly found to migrate slightly more to the left in the fingerprint of homogeneously labeled RNA than it does in the fingerprint of the in vitro-labeled RNA. Other examples are oligonucleotides 33A and

and previous analysis of Akv virus (29) indicated that some spots of the fingerprint corresponded to two oligonucleotides present in about equal amounts. Such oligonucleotides include 19A and

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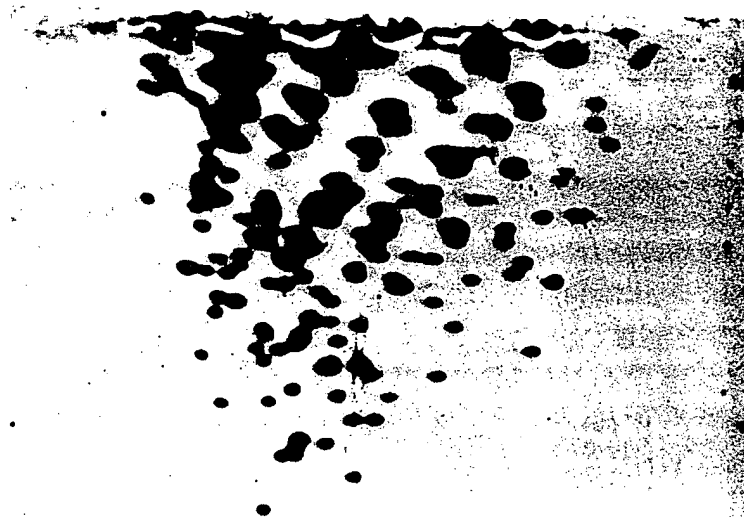


FIG. 2. RNase  $T_1$  fingerprint of 70S RNA of Akv(I) homogeneously labeled by  $^{32}\text{P}$ . The RNase  $T_1$  digestion was carried out after the addition of nonradioactive carrier RNA as described in the text.

33B, which are separated in this fingerprint but which overlap in the fingerprints of Fig. 1. Oligonucleotides 61A and 61B are resolved from each other in the fingerprint of homogeneously labeled RNA but not in the fingerprint of the RNA that was labeled in vitro.

Such minor differences might be explained by the slight differences in the structure of the oligonucleotides analyzed by the two methods. RNase  $T_1$  digestion of homogeneously labeled RNA will produce an oligonucleotide that has a 5'-hydroxyl group and a 3'-terminal phosphate. In the procedure used for 5' end labeling, oligonucleotides contain a 5'-terminal phosphate and a 3'-terminal hydroxyl group. Some minor differences in the patterns are also observed among the oligonucleotides resolved as discrete spots in the dense upper middle region of the fingerprint. Due to the complexity of the pattern in this region, it is not clear whether all of these differences represent slight mobility changes or oligonucleotides unique to one or the other fingerprint. We conclude, however, that all of the well-resolved oligonucleotides of the homogeneously labeled RNA are found in the pattern of 5'-labeled oligonucleotides and that no good candidate for the oligonucleotide of the 5' end of the viral RNA can be identified.

We note that some of the discrete spots of Fig. 1 are probably underdigestion products. These include 2A and 48, which are present in nearly unimolar amounts, and a number of more weakly labeled oligonucleotides (for example, the two oligonucleotides between no. 8 and 13A):

We have found that denaturation of the RNA by heating to  $90^\circ\text{C}$  for 5 min before RNase digestion leads to a complete disappearance of oligonucleotides 2A and 48, as well as most of the minor oligonucleotides (Fig. 3). On this heavily exposed autoradiogram only a few minor extra oligonucleotides appear. The most heavily labeled one is an oligonucleotide between no. 8 and 13A. This indicates that oligonucleotides 2A and 48, as well as some weak oligonucleotides, represent incomplete digestion products containing RNase  $T_1$  cleavage sites, which are protected by the secondary structure of the non-denatured RNA. In agreement with this result, we have found that oligonucleotide 2A isolated from the second-dimension gel contains several RNase  $T_1$  cleavage sites.

Fingerprints of very small amounts of RNA can be obtained by this method. Figure 4 is a fingerprint of Akv RNA derived by digestion of 2 ng of 70S RNA (approximately  $10^{-15}$  mol). Digestion was in 2  $\mu\text{l}$  of buffer, and the RNA was labeled in 3  $\mu\text{l}$  of the kinase reaction. The second-dimension gel contained 7 M urea to permit the exposure of the autoradiogram at  $-70^\circ\text{C}$  with intensifying screens. The inclusion of 7 M urea in the gel accounted for minor changes in the mobility of some of the oligonucleotides (compare Fig. 1 and 4). Incomplete digestion products such as spot no. 2A and 48 were much weaker or absent when RNase digestion was carried out at a very low RNA concentration (compare Fig. 1 and 4). This result demonstrated that the RNase exhibits the same specificity at this low concen-

FIG. 3. 70S RNA (as described in sample was and subse film was present in

FIG. 4. R carried out. Urea was in

tration of RNA and that the incomplete digestion products are less pronounced at this concentration of RNA. This would also explain the absence of these oligonucleotides from the fingerprint of homogeneously labeled RNA as this digestion was carried out at a low concentration of viral RNA. The significance of this protection of RNase cleavage sites is not clear. Presumably, it represents interactions between two or more

70S structures as the protection was found to be dependent upon the concentration of RNA.

The above results demonstrate that the technique of carrier-free digestion of small quantities of RNA, followed by 5' labeling of the oligonucleotide, leads to the detection of all RNase T<sub>1</sub>-resistant oligonucleotides and that these are labeled uniformly. Incomplete digestion products may be present when a high concentration of RNA is used, but these products can easily be identified and avoided by dilution or denaturation or both of the sample.

To determine whether all of the unique oligonucleotides detected above are derived from the viral genome, rather than from cellular RNA copurified with 70S RNA, we isolated 35S viral RNA and analyzed it by the same technique. Figure 5 shows that all of the RNase T<sub>1</sub>-resistant oligonucleotides that were assigned a number in Fig. 1b were present in the fingerprint of 35S RNA. This demonstrated that the 70S RNA isolated by the micromethod described above was free from any significant contamination of sequences of nonviral origin. For this experiment, Akv(S) produced by the AKR fibroblast line was used. Note that the fingerprint of this RNA differs from that of Akv(I) by the presence of an oligonucleotide (1\*) to the left of oligonucleotide 1. This difference is also present in the fingerprint of Akv(S) 70S RNA. The nucleotide sequence of this novel oligonucleotide differs from the sequence of oligonucleotide 1 in only

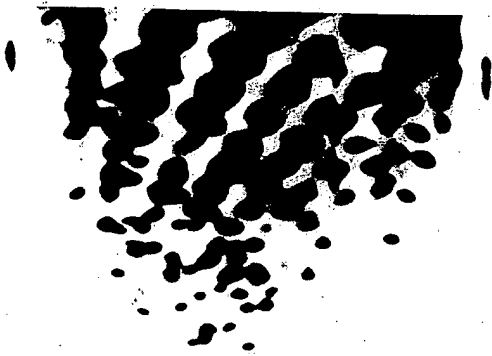


FIG. 3. RNase T<sub>1</sub> fingerprint of heat-denatured 70S RNA of Akv virus. The experimental details were as described in the legend to Fig. 1, except the RNA sample was heated to 90°C for 5 min in 40  $\mu$ l of water and subsequently lyophilized before digestion. The film was overexposed to detect all oligonucleotides present in less than molar quantities.

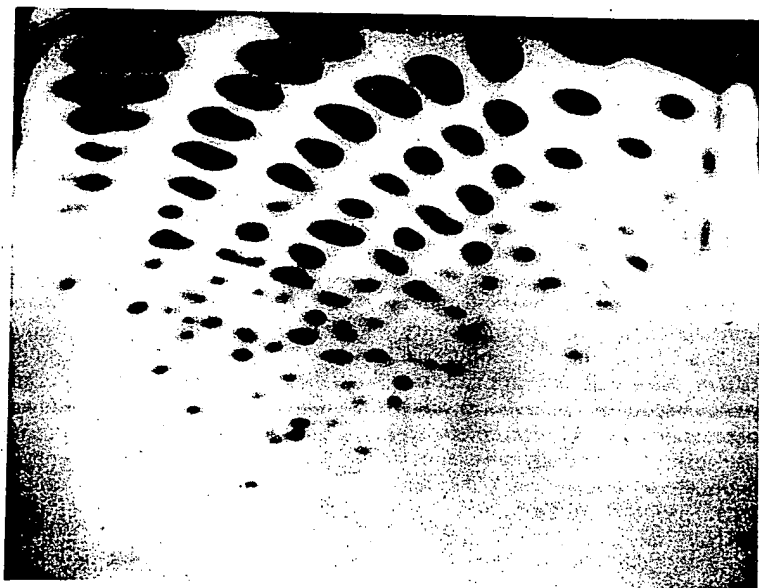


FIG. 4. RNase T<sub>1</sub> fingerprint of 70S RNA of Akv virus, using 2 ng of RNA. The RNase T<sub>1</sub> digestion was carried out in a 2- $\mu$ l volume, followed by labeling in 3  $\mu$ l of the polynucleotide kinase reaction mixture for 4 h. Urea was included in the second-dimension gel to permit long-term exposure at -70°C.

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FIG. 5. RNase  $T_1$  fingerprint of 35S RNA of Akv(S). The conditions for the digestion and labeling of RNA were as described in the legend to Fig. 1.

the 5'-terminal position (see Table 3).

**Nucleotide sequence analysis.** To further characterize the Akv virus, we wished to determine the nucleotide sequence of unique RNase  $T_1$ -resistant oligonucleotides purified by the two-dimensional electrophoresis system. Recently, a number of techniques for sequence analysis of terminally labeled RNA have been developed; therefore, such an analysis was feasible (10, 16, 19, 24).

Since the RNase  $T_1$ -resistant oligonucleotides to be analyzed were less than 30 nucleotides long and lacked internal guanosine residues, the presence of strong secondary structures was unlikely. Therefore, we decided to adapt a method based upon mapping of cleavage sites for sequence-specific RNases under mild denaturing conditions (10). The procedures described above permit all of the cleavage reactions to be done under similar conditions. The cleavage products are mapped by one-dimensional gel electrophoresis. These two features make this method suitable for the simultaneous sequence analysis of large numbers of oligonucleotides. The complete sequence analysis can be carried out with about 10,000 dpm/oligonucleotide, an amount of radioactivity which can be obtained from less than 100 ng of RNA by the procedures described above.

The cleavage specificities of the RNases used for the sequence analysis are listed in Table 2. RNases A,  $U_2$ , and  $T_2$  are highly purified commercial preparations, whereas the *P. polyceph-*

*alum* RNase preparation used here is a crude preparation of extracellular enzymes (see above). We find that this enzyme preparation (as well as the preparation donated by H. Donis-

Keller) will cleave all  $Ap^{\perp}N$  and  $Up^{\perp}N$  bonds, whereas purified enzyme preparations have a more restricted specificity (26). Although the preparation used here contains all extracellular enzymes, the contamination by other RNases is insignificant under these reaction conditions.

For the analysis of the Akv virus genome, the unique RNase  $T_1$ -resistant oligonucleotides were eluted from second-dimension gels in the presence of nonradioactive carrier RNA and divided into samples which were incubated with the various RNases under conditions for partial digestion as described above. The cleavage products were separated according to chain length by electrophoresis in denaturing polyacrylamide gels at neutral pH, and the products containing the radioactive phosphate group of the 5' end were visualized by autoradiography. Examples of such autoradiograms are shown in Fig. 6. The nucleotide sequences were determined from the autoradiogram by using the enzyme specificities given in Table 2. Briefly, the sequence deduction includes a determination of the chain length from the RNase  $T_2$  track, mapping of the adenosine residues by inspection of the RNase  $U_2$  track, and discrimination of pyrimidine residues by inspection of the *Physarum* RNase track. The RNase A tracks serve to support the sequence identification by using the range of cleav-

TAB

Enzyme

RNase  $U_2$ 

RNase A

*Physarum*  
RNasesRNase  $T_2$ 

\* All of the RNase

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TABLE 2. Cleavage specificity of RNases used for nucleotide sequence analysis

Enzyme	Cleavage specificity	Comments <sup>a</sup>	Reference
RNase U <sub>2</sub>	Ap-N	Low frequency of cleavage at ↓ Ap-G at the 3'-terminal residue	26; our unpublished observation
RNase A	Pyp-N	Range of susceptibility of bonds ↓ Cp-A > Up-A >> Cp-C > ↓ Up-C >> Cp-U > Up-U	14
Pharysarum RNases	Ap-N, Up-N	Ap-N bonds more susceptible than ↓ Up-N bonds; high susceptibility ↓ of Up-G and Ap-G at the 3' end of oligonucleotides; also weak ↓ cleavage of Cp-G at the 3' terminus	Unpublished observation
RNase T <sub>2</sub>	Np-N		17

<sup>a</sup> All of the RNases used here seem to have a somewhat altered specificity for cleavage at the 5' terminus.

age specificity given in Table 2.

As we found that the unambiguous determination of the 5'-nucleotides by this procedure was difficult, we identified the terminal nucleotide by a different procedure. The 5'-labeled oligonucleotides were subjected to complete alkaline hydrolysis, and the 5'-nucleotide was identified by thin-layer chromatography as shown in Fig. 7, using radioactive 5',3'-nucleoside diphosphates as markers. We found that a 5'-terminal cytidine residue was detected as a doublet by this procedure. This doublet was also produced by the complete hydrolysis of 5'-labeled polycytidylic acid.

Using these procedures, we determined the nucleotide sequences of 75 RNase T<sub>1</sub>-resistant oligonucleotides of the genome of Akv virus (Table 3). The sequences given were based upon at least two determinations of independently isolated oligonucleotides. The unique oligonucleotides of the genome of Akv virus detected in previous studies (29) could be identified among those characterized here. In the right column of Table 3, the oligonucleotide numbers used in previous studies are listed together with their major RNase A digestion products determined in these studies (29). A detailed comparison of the results of the partial and complete sequence analyses shows that there is good agreement between the two sets of data. For the well-re-

solved oligonucleotides, less-than-complete agreement between the results of the partial and complete sequence analyses (e.g., no. 2 and 5) can be explained by inaccuracies associated with the method of partial sequence analysis, since the precise identification of RNase A digestion products of three or more adenosine residues by this method is difficult. For the less-well-resolved oligonucleotides, the comparison shows that all of the RNase A digestion products predicted from the sequences have been detected in the previous analysis. However, for a few oligonucleotides (no. 4A, 30, 31, 41, and 55) the previous partial sequence analysis of Rommelaere and co-workers (29) led to the detection of additional RNase A digestion products. It seems likely that these few extra products which do not correspond to the sequences determined here are derived from contaminating neighboring oligonucleotides.

Another verification of the nucleotide sequences can be gained from an inspection of the mobilities of the oligonucleotides in the two-dimensional electrophoresis system. This system separates the oligonucleotides according to base composition and size. In this system there should be a simple relationship between the mobility of an oligonucleotide and its base composition. When we correlated the base compositions calculated from the sequences of Table 3 with the

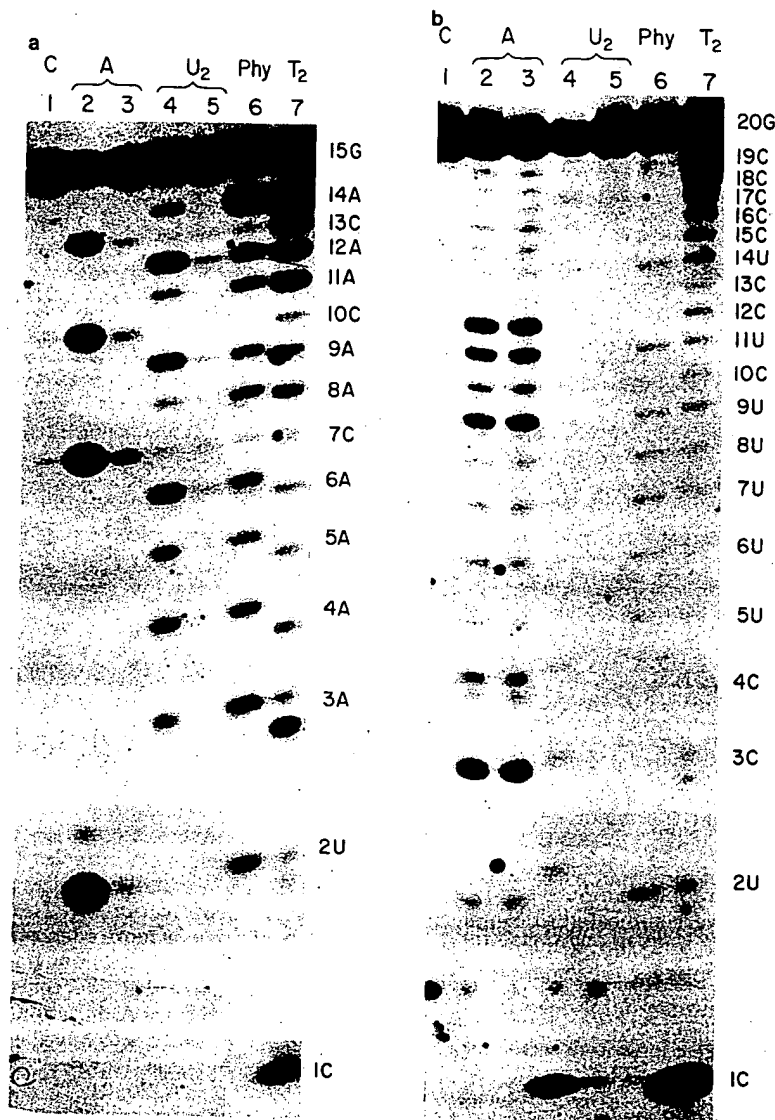


FIG. 6. Examples of autoradiograms of gels used for nucleotide sequence analysis. (a) Oligonucleotide 25; (b) oligonucleotide 17. Samples of the 5'-labeled oligonucleotides were digested with RNase A, RNase U<sub>2</sub>, Physarum RNase, and RNase T<sub>2</sub>, and the fragmentation pattern was analyzed by one-dimensional gel electrophoresis as described in the text. The identification of the 5'-terminal nucleotide is based upon a separate analysis (see the text and Fig. 7). Note that the weak band in the Physarum RNase track at position 19 of oligonucleotide 17 is identified as a cytidine residue, whereas the adenosine in the equivalent position 14 of oligonucleotide 25 gives rise to a strong band (see comment in Table 2). Also note in the analysis of oligonucleotide 17 that the strong bands in the RNase A tracks represent the cleavage of bands which are followed by a cytidine residue. The radioactive material located between nucleotides 1 and 2 of this autoradiogram represents an unknown contaminant.

positions of the oligonucleotides in the fingerprint, we found the expected correlations between mobility and base composition. The oligonucleotides (e.g., no. 27, 80, 81, 88, and 89) of the vertical isopyth in the left part of the pat-

tern (Fig. 1b) contain no uridine residues. The next isopyth containing oligonucleotides 34, 35, 36, and 76 contain oligonucleotides of one uridine residue and so on. In the middle of the fingerprint the pattern is more complex; e.g., the

FIG. 7. *lucose thin layer chromatography (TLC) of oligonucleotides. The oligonucleotides were labeled with <sup>32</sup>P at the 5' end and separated on a TLC plate. The bands are labeled with nucleotide sequences: 19C, 18C, 17C, 16C, 15C, 14U, 13C, 12C, 11U, 10C, 9U, 8U, 7U, 6U, 5U, 4C, 3C, 2U, and 1C.*

isopyth containing oligonucleotides 63.

The expected correlations between mobility and base composition within the fingerprint are consistent with the content of oligonucleotides in the left part of the fingerprint. For example, the oligonucleotide 19C (residue 19) is identified as a cytidine residue, whereas the adenosine in the equivalent position 14 of oligonucleotide 25 gives rise to a strong band (see comment in Table 2). Also note in the analysis of oligonucleotide 17 that the strong bands in the RNase A tracks represent the cleavage of bands which are followed by a cytidine residue. The radioactive material located between nucleotides 1 and 2 of this autoradiogram represents an unknown contaminant.



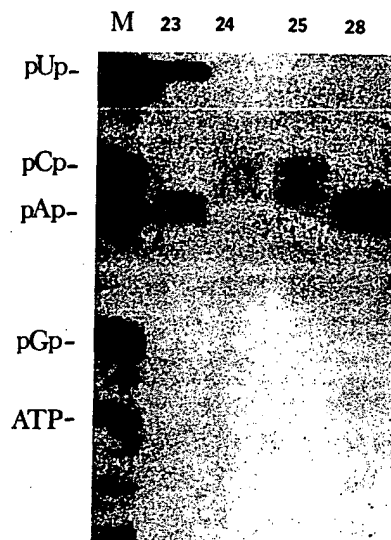


FIG. 7. Autoradiogram of polyethyleneimine cellulose thin-layer chromatogram developed in 0.5 M  $\text{KH}_2\text{PO}_4$  (pH 3.5), illustrating the method used for the determination of 5'-terminal nucleotides. The origin of the chromatogram is at the bottom. The lane marked with an "M" contains a mixture of 5',3'-nucleoside diphosphates. The numbers of the oligonucleotides used for the analysis are shown at the top. The assignments of the 5'-nucleotides are as follows: no. 24, cytidine; no. 25, cytidine; no. 28, adenosine; no. 23, a mixture of two oligonucleotides, one carrying a 5'-terminal adenosine residue and the other carrying a 5'-terminal uridine residue.

isoplyth of six uridine residues contain oligonucleotides 2, 98A, 98B, 99A, 99B, 8, 18, 47, 56, and 63.

The exact location of the oligonucleotides within the isoplyth is dependent upon its relative content of cytosine and adenosine. The cytidine-rich oligonucleotides are found within the upper left part of the isoplyth, and the adenosine-rich oligonucleotides are found in the lower right part. For example, oligonucleotide 28 (1 uridine residue, 10 adenosine residues, and 2 cytidine residues) is found next to oligonucleotide 29 (2 uridine residues, 9 cytidine residues, and 1 adenosine residue). The use of these rules makes it possible to determine whether the uridine content of an oligonucleotide is consistent with its position in the fingerprint. The migration differences associated with the variation in the relative content of cytidine and adenosine are less pronounced, but also useful in this control. With only two exceptions, the mobilities of all oligonucleotides were those predicted by the above rules from their length and base composition as determined from Table 3. The first exception

was oligonucleotide no. 49 (14 nucleotides long), which was found next to oligonucleotide 55 (13 nucleotides long). However, we found that oligonucleotide 49 moved relatively slower in the second dimension when the electrophoresis was carried out at a higher temperature. This variation in mobility can be explained by the presence of a secondary structure of this oligonucleotide. One such structure containing three adenosine-uridine base pairs and one cytidine-guanosine base pair can be predicted from the nucleotide sequence (Table 3). The second exception involved the relative positions of oligonucleotides 34 and 35. These two oligonucleotides were found to be of identical chain length. Although oligonucleotide 35 was found to the upper left of oligonucleotide 34, the determined sequence indicates that no. 35 is richer in adenosine and poorer in cytidine residues than is no. 34. The reason for this discrepancy is not clear.

With the exception of this one case, we found that all of the oligonucleotide sequences determined here were consistent with the migration of the oligonucleotides, as well as with the published nucleotide sequence information for Akv virus. We therefore believe that the rapid method of partial RNase digestion, followed by one-dimensional electrophoresis, leads to the determination of correct sequences for RNase  $T_1$ -resistant oligonucleotides.

## DISCUSSION

In this study we analyzed the RNase  $T_1$  fingerprints of the genome of Akv virus. The two virus isolates studied here were identical except for a minor heterogeneity in Akv virus spontaneously produced by an AKR embryo fibroblast cell line. This heterogeneity, detected as the presence of two oligonucleotides of related nucleotide sequence (no. 1 and no. 1\*), may represent a slight difference between the provirus of the two loci *Akv-1* and *Akv-2*. This variation may also represent a special feature of the particular cell line used rather than a reproducible difference between the two loci. We also analyzed the genome of AKR pII, a cloned Akv isolate derived by Nowinski and co-workers (22), and found that the RNase  $T_1$  fingerprints of this isolate are indistinguishable from the Akv(I) fingerprint.

In a previous study, Rommelaere et al. (29) analyzed the genome of endogenous AKR virus at the two loci *Akv-1* and *Akv-2* and found that the two viruses were indistinguishable. The oligonucleotide pattern of the virus shown here contains all of the unique oligonucleotides analyzed by these authors. However, due to the improved two-dimensional gel electrophoresis

oligonucleotide 25; A, RNase  $U_2$  dimensional gel based upon a track at position valent position the analysis of ends which are and 2 of this

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TABLE 3. Nucleotide sequence of RNase T<sub>1</sub>-resistant oligonucleotides of *Aku* virus<sup>a</sup>

Oligo-nucleotide no.	Nucleotide sequence	Chain length	Previous analysis (29)	
			Oligo-nucleotide no.	Major RNase A digestion products
1	UCUCUCCCAAACUCUCCCCCUCUCCAACG	29	1	(A <sub>3</sub> C), (A <sub>2</sub> C)
1*	ACUCUCCCAAACUCUCCCCCUCUCCAACG	29		
2	CUCUUACCCCCUCUAUAAAACCCAG	25	2	(AC), (AU), (A <sub>3</sub> C), (AG)
3	UCCUCACCACCCACCAG	17		
4A	UCCCUAAAACCCAUCAAG	18	3	(AC), (AU), (A <sub>2</sub> C), (A <sub>2</sub> G)
4B	ACAAAACCCUCCAUAUG	18	4	(AC), (AU), (A <sub>3</sub> C), (A <sub>2</sub> U)
5	UCCAUA AAAACAUAACCCCAUG	21	5	(AC), 2(AU), (A <sub>3</sub> C), (A <sub>2</sub> U)
6	AUACAUCACUCUCCUUAUAG	21	6	3(AC), 3(AU), (AG)
7	CCACCUUACUCCUCCUCCG	20	7	2(AC)
8	UAUCUCAACCAUACUUG	20	8	2(AU), 2(AC), (A <sub>2</sub> C)
9	UACCUACUCCAACCAUACUUG	23	9	3(AC), (AU), (A <sub>2</sub> C)
10	ACACCCUACCCUCCCG	19	10	3(AC)
11	CCUUCAAACUCCACCCCAACCG	22	11	(AC), (A <sub>2</sub> C), (A <sub>3</sub> C)
12	CUCCCAUCCUCCCAUCCG	18	12	2(AU)
13A	CAACUCCUUAACUAUCCG	18		
13C	AYUCUAAACUAAAYUAACG	18		
15	AAAAUAAUUAUCCUCCUUCUCUG	25	15	2(A <sub>2</sub> U), (A <sub>3</sub> U)
16	UCACUCACUCUUCUCCUCCAG	21	16	2(AC), (AU)
17	CUCCUUUUUCUCCUCCCG	20	17	No A's
18	AUCUACUAUUCUAAAAAG	18	18	1-2(AC), 2(AU), (A <sub>3</sub> G)
20	ACUUCUAUAUACUCCCG	17	20	2(AC), 2(AU)
21	CAACAAUCUAACCUACG	17		
24	CCCUCAACCUACCCAG	16		
25	CUAAAACAACAACAG	15	14	0-1(AC), 2(A <sub>2</sub> C), (A <sub>2</sub> C), (AG)
27	ACCACCCCCACCG	13		
28	AAAUCAAAAACAAG	14		
29	CAACCUCCACCCUG	15		
30	AUAAAACCAUCAUG	14	28	(AC), 3(AU), (A <sub>2</sub> C)
31*	UUCCAACAACUCCAG	15	35	0-1(AC), 0-1(AU), 2(A <sub>2</sub> C), (AG), (AU), (AG)
32	CCCCUCUCCCAUAG	15	27	
33	ACACCUAUAACCCAG	14		
34	AACCCACACUCG	13	13	3(AC), (A <sub>2</sub> C)
35	AACCAACCAACUG	13		
36	AAAUCAAACAG	11		
37A	CUCACCCACCUUG	13		
40A	ACCCUCUAAAACCUUAG	16		
41	CCACCUCCACUUCUG	15	29	2(AC), (AU)
42	AUAUCCACCAUCAUG	15		
43	UUAACUAAACUAAAAG	16		
44	UCCAUCACUAAUCUAG	16	19	(AC), (AU), (A <sub>2</sub> U), (AG)
46	UUUUUAACCUACCCUG	16	23	(AC), (A <sub>2</sub> C)
47	UUUACCACCAAUUUG	15	22	2(AC), (A <sub>2</sub> U)
49	AUCUUUAUAAAAG	14		
50	CCUAUUUCCAUAUACUG	18	24	(AC), 3(AU)
51A	CUUCUAAAAACAUG	14		
52	AAUUAACUAAUAG	13		
54	CUUUCACACUCCG	13		
55	AUCUAUAUAACUG	13	21	(AC), 2(AU), (A <sub>2</sub> C)
56	AAUCCUUCUAUUG	13	25	(AU), (A <sub>2</sub> U)
57	UUAUUUUUACUCUUUG	16	33	(AC), (A <sub>2</sub> U)
58	UACUUUUUAUUUG	13	30	(AC), (A <sub>2</sub> U)
59	CAUCUACUUUUUG	13		
60	UUUUCUCCUUCUG	13		
62	UUAUUCARCUG	11	32	2(AU)
63	AUUUUUAUUCAG	11	26	2(AU), (AG)
66A	UUCUCUACUG	10		

Oligo-nucleotide no.

67

68

69

70A

70B

73

74

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76

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98A

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<sup>a</sup> Abbrev.<sup>b</sup> This samsystem u  
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TABLE 3—Continued

Major RNase A digestion products	Oligonucleotide no.	Nucleotide sequence	Chain length	Previous analysis (29)	
				Oligonucleotide no.	Major RNase A digestion products
1), (A <sub>2</sub> C)	67	UUCUUUUAG	9		
1), (AU), (A <sub>2</sub> C), (G)	68	AAUUUUUUG	8		
	69	UUUCUAUG	8		
	70A	UUUUUCG	7		
	70B	CUUUUUG	7		
1), (AU), (A <sub>2</sub> C), (G)	73	UUUCAAAAACAG	12		
1), (AU), (A <sub>2</sub> C), (G)	74	UUCACCACCCUG	12		
	75	AUAUAAAAAG	10		
	76	UCUCCAAAACG	11		
1), 2(AU), (A <sub>2</sub> C), (G)	78	AAUAAAAAG	8		
2), 3(AU), (AG)	79	CCCCCCCCUG	10		
	80	AAAAACAAG	9		
U), 2(AC), (A <sub>2</sub> C)	81	ACCCCCCG	9		
C), (AU), (A <sub>2</sub> C)	83	ACCAAAAAG	8		
	88	CCCCCACCAG	10		
	89	CCAAACCCCG	10		
1), (A <sub>2</sub> C), (A <sub>2</sub> C)	98A	CUCUCAAAACCCUUUAAAAAUCAG	24		
U)	98B	ACUCAACAAUAUCAACUUARG	24		
	99A	CYCAAUCCUUAUAACCUCCUAG	23		
	99B	CCUCAUUUAAACCUAACCAUACAG	23		

\* Abbreviations: A, adenosine; C, cytidine; U, uridine; G, guanosine; Y, cytidine or uridine; R, unknown residues.

\* This same oligonucleotide sequence has been determined in the genome of a closely related virus (28).

system used here, we detected a number of unique oligonucleotides not previously analyzed.

In the course of these studies, we encountered persistent problems in the maintenance of homogeneous stocks of Akv virus. The fingerprint patterns of the RNA were not constant. Upon continued passage of persistently infected cell lines, oligonucleotides that were present in the genome in unimolar amounts were greatly decreased and other oligonucleotides appeared. Some of the new oligonucleotides appeared in virus stocks that had independent passage histories. Such changes were noted with viruses harvested from AKR cells, as well as with cloned viruses propagated in NIH 3T3 cells. The viruses used for the analysis here were obtained from stocks of viruses that did not have long tissue culture passage histories. A hint of this problem was observed in the fingerprint of the 35S RNA of the virus that was spontaneously produced by an AKR cell line (Fig. 5). Oligonucleotides 8 and 9 were present in less than molar quantities. We found that virus derived by chemical induction of non-virus-producing AKR cells that was propagated on NIH 3T3 cells was the best source of homogeneous RNA.

sequence of 75 unique RNase T<sub>1</sub>-resistant oligonucleotides of the genomes of Akv virus. To assign some of these sequences to specific viral genes, we correlated the nucleotide sequences obtained here with the amino acid sequences of virus-coded proteins available in the literature (23). These sequences include the N-terminal sequences of *gag* gene-coded proteins p12, p30, and p10. The match between the amino acid sequence and the sequence of the RNA are listed below.

	14							19
	p30	Pro	- Phe	- Ser	- Ser	- Ser	- Ser	Asp
Oligonucleotide	60	G	UUU	UCC	UCC	UCU	G	

The amino acid sequence of p30 in positions 13 to 18 corresponds to the nucleotide sequence of oligonucleotide no. 60. The NH<sub>2</sub>-terminal amino acid sequence of p12 corresponds to the sequence of oligonucleotide 2, except our nucleotide sequence would predict an isoleucine residue instead of leucine at position 7. The identification of the adenosine residues in the first position of the isoleucine codon is certain in our determination.

	1									10	
	p12	Pro	- Ala	- Leu	- Thr	- Pro	- Ser	- Ile	- Lys	- Pro	- Arg
Oligonucleotide	2	GCU	CUU	ACC	CCC	UCU	AUA	AAA	CCC	AG	

In this study we determined the nucleotide

Whether this difference is due to an error in the

amino acid sequence analysis or to a difference between the strains of virus used is not clear. An isoleucine residue in position 7 has been found in p12 of a closely related virus, radiation murine leukemia virus (S. Orozlan, personal communication).

The homologs of virion proteins p30 and p10 of different strains of murine leukemia virus are known to share structural similarities (3, 23). Since extensive amino acid analysis of the proteins has been carried out for Rauscher murine leukemia virus (S. Orozlan, personal communication), we analyzed our oligonucleotide sequences for a possible correspondence with the amino acid sequence of p30 and p10 of this virus. The amino acid sequence of positions 19 to 23 of Rauscher murine leukemia virus p30 corresponds to the sequence of oligonucleotide 55, as follows:

	19					23
	p30 Asp	- Leu	- Tyr	- Asp	- Trp	
Oligonucleotide	55 GAU	CUA	UAU	AAC	AG	

It is noteworthy that this overlap would place oligonucleotide 55 next to oligonucleotide 60 in the genome. The nucleotide sequence of oligonucleotide 49 corresponds to an amino acid sequence of the C-terminal region of p30, as follows:

	p30 Lys	- Ile	- Thr	- Asp	- Lys	- Arg
Oligonucleotide	49 G	AUC	UUU	AAU	AAA	AG

In agreement with these overlaps, we have found that oligonucleotides 2, 49, 55, and 60 are all derived from the 5' part of the genome (17a; unpublished observations), the region expected to carry the *gag* gene.

The methods used here for the analysis of the genome of Akv virus are generally useful for the analysis of RNA in small quantities. Conditions are defined for the specific RNase T<sub>1</sub> digestion of RNA of a high complexity in nanogram quantities and for the uniform labeling of the digestion products. The high-resolution gel electrophoresis system described provides the resolution of a large number of unique oligonucleotides into a pattern characterized for that particular RNA species. Furthermore, we describe a procedure for the isolation of small quantities of pure viral RNA from retroviruses. This isolation procedure should be generally applicable for the isolation of RNA from viruses containing a 3'-terminal polyadenylic acid sequence of the genome. The requirement of only nanogram amounts of RNA for these methods makes them suitable for studies of a variety of problems in the biology and classification of RNA viruses grown in tissue culture or isolated directly from

animal tissues. For example, we have used these methods in the study of the genomic variation of influenza virus (8).

The 5'-terminal location of the radioactive label makes subsequent characterization of the unique oligonucleotides by a partial or complete nucleotide sequence analysis feasible. In this study we used the method based upon the analysis of products produced by partial digestion with sequence-specific RNases. As described here, the method permits the nucleotide sequence analysis of 50 to 80 unique oligonucleotides in RNA of a nonredundant sequence of about 10,000 nucleotides. Under optimal conditions this analysis could be carried out with 20 to 100 ng of starting material. The sequencing method used here has been selected because of its simplicity, which makes it possible to analyze all unique RNase T<sub>1</sub>-resistant oligonucleotides of one fingerprint in parallel. Although the method used here may have an error frequency higher than more elaborate methods, we find that nucleotide sequences determined by this procedure are useful for the following: (i) correlating amino acid sequences and nucleotide sequences as discussed above; (ii) detecting identical or related oligonucleotide sequences in different RNA samples (17a, 31); and (iii) correlating oligonucleotide sequences with the results of DNA sequence analysis.

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